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Molecular and Cellular Analysis of Chlamydia Trachomatis: Persistence and Reactivation

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MOLECULAR AND CELLULAR ANALYSIS OF CHLAMYDIA TRACHOMATIS PERSISTENCE AND REACTIVATION

A Dissertation

presented to

the Faculty of the Department of Microbiology

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In Partial Fulfillment

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Doctor of Philosophy in Biomedical Sciences

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by Kimberly R. Tau

May 1992

APPROVAL

This is to certify that the Graduate committee of

Kimberly R. Tau

met on the

30th day of March, 1992

The committee read and examined her dissertation, supervised her defense of it in an oral examination, and decided to recommend that her study be submitted to the Graduate Council and the Associate Vice-President for Research and Dean of the Graduate School, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Sciences.

Chair, Graduate Committee

Signed on behalf of
the Graduate Council

Associate Vice-President for Research
and Dean of the Graduate School

MOLECULAR AND CELLULAR ANALYSIS OF CHLAMYDIA TRACHOMATIS PERSISTENCE AND REACTIVATION

Molecular and Cellular Analysis of Chlamydia trachomatis
Persistence and Reactivation

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Chlamydia trachomatis (CT) is the most prevalent sexually-transmitted infection in the United States. It has been suggested that CT infections can become latent. This has not been substantiated. CT persistence was examined at the molecular and cellular level in vitro and in vivo.

Penicillin treatment of CT in vitro results in abnormal inclusions and reduced recovery of infectious CT. Penicillin did not inhibit initial stages of infection, but did downregulate CT rRNA levels after 25 hours post-inoculation (p.i.). DNA amplification was employed to differentiate between a resolved infection and a persistent one. Utilizing a primer pair that amplified a 144 bp fragment in the CT MOMP gene, CT-persistently-infected McCoy cells maintained in penicillin medium were examined. Though undetectable by other assay methods, these cells harbored the CT genome for 18 passages. Removal of penicillin 1, 3 or 6 passages p.i. and subsequent cultivation in permissive medium resulted in "recovery" to productive infection. Removal of penicillin at later passages resulted in low level inclusion formation but no infectious progeny. Penicillin treatment in vitro resulted in a persistent infection undetectable by most methods.

Female C_3H/HeNCRL mice were inoculated with CT intrauterinely and intravaginally in two separate experiments. In one, CT infection was established in untreated and Depo-Provera (DP)-pretreated mice. DP pretreatment enhanced vaginal shedding of infectious CT. A negative vaginal culture did not correlate with elimination of CT from the upper tract. In the second, penicillin therapy did not halt vaginal CT shedding, however, it reduced frequency of recurrent vaginal CT shedding. To examine reactivation, culture-negative mice ($\geq$2 successive vaginal cultures) were injected with cortisone-acetate (CA) or DP; mice from same subpopulation injected with saline served as controls. Transient vaginal CT shedding was reactivated in penicillin-treated mice (14% CA-injected), and in unmedicated mice (28% CA-injected, 33% DP-injected). Saline injection did not reactivate vaginal CT shedding. At time of sacrifice (16 or 22 weeks p.i.) no infectious CT was detected in upper tract tissues, although tissue damage was observed in most mice (70-71%). It is unknown if these mice harbored a persistent infection undetectable by culture. Further work utilizing molecular techniques is needed to resolve this question.

DEDICATION

This dissertation is dedicated to my loving family and friends without whose love, support and empathy this dream would never have been realized.

ACKNOWLEDGMENTS

I owe a great deal of gratitude to numerous people without whose assistance and advice, this dissertation would never have been completed. First I would like to thank my mentor, Dr. Bill Campbell, who believed in me from the start and whose guidance and training have been indispensable. I would also like to thank the other members of my committee whose expertise and advice made this such a worthwhile educational experience: Dr. Jill Suttles, Dr. Bob Stout, Dr. Katie-Jane Mayberry-Carson and Dr. Scott Champney. Your input and experience have been invaluable (You're right, Jill. Shout does remove bromophenol blue from cotton shirts.). A special note of thanks goes to my research colleagues, Tove, Penny, He, Tina and Bojun, who have been delightful friends and co-workers. An extra special thanks goes to Dr. Bill Mayberry whose timely advice on science and those wretched beasts called computers has saved my sanity on numerous occasions. I would also like to thank Janette Taylor for her help in preparing this manuscript. Special thanks also go to my Mom, Dad, Bren and Merriam whose loving support and willingness to put up with my moments of "panic attack" helped to keep me going, even when the going got rough(er). Finally I would like to thank the one who was almost always there when I needed him (even though he wouldn't learn tissue culture): Terry Sanders, without whom this would never have been achieved.

CONTENTS

LIST OF TABLES

Table — Page

LIST OF FIGURES

CHAPTER 1

Introduction

General Characteristics of the Genus Chlamydia

The Chlamydiae belong to a unique genus of obligate intracellular prokaryotes that invade eukaryotic cells, parasitizing high energy compounds and other nutrients. The members of the genus share certain traits including an obligate intracellular habitat, a developmental cycle with two morphologically distinct forms, an inability to synthesize high energy compounds, and a very small genome (660 MDa) (Moulder 1988). The genus currently contains three recognized species: Chlamydia psittaci, Chlamydia trachomatis (CT) and Chlamydia pneumoniae. C. psittaci is a recognized cause of diverse veterinary and human disease states including abortion in sheep and cattle, pneumonia, airsacculitis, arthritis, polyarthritis-polyserositis, urogenital infections, mastitis, intestinal infections, enteritis in ruminants and chlamydiosis in wild and domesticated birds (Storz 1988). C. psittaci is less host- and tissue-specific, with less homogeneity within the species than is CT. CT is composed of three biovars (distinctive variations between members of the same species determined by biological differences): lymphogranuloma venereum (LGV), trachoma inclusion conjunctivitis agents (TRIC), and mouse pneumonitis (MoPn). LGV is a highly invasive pathogen with a tropism for lymphoid tissue. LGV is the etiologic agent of lymphogranuloma venereum, a sexually-transmitted disease that is fairly rare in the United States, but is far more common in other parts of the world including Southeast

Asia, India, and Africa (Schachter 1988). The TRIC agents cause a variety of disease including trachoma, the leading cause of preventable blindness in the world. There are eighteen serovars of CT, four of which are associated with endemic trachoma (A, B, Ba and C), four with LGV (L1, L2, L2a and L3); the other serovars are primarily associated with genital and neonatal infections (Freeman 1985, Wang and Grayston 1991). There is nearly 100% DNA homology shared by the LGV and TRIC biovars, even though each causes distinctly different diseases (Moulder 1988). In contrast, MoPn, the only nonhuman biovar of CT, shares only 30-60% DNA homology with the other biovars of CT (Moulder 1988). MoPn, the murine biovar of CT generally causes a pneumonia, but has been used to establish genital infections experimentally. C. pneumoniae has been determined to be an important human pathogen, causing pneumonia in adults as well as neonates. It is biochemically distinct from C. psittaci, the species in which it was once classified (Patton, personal communication).

Developmental Cycle of CT

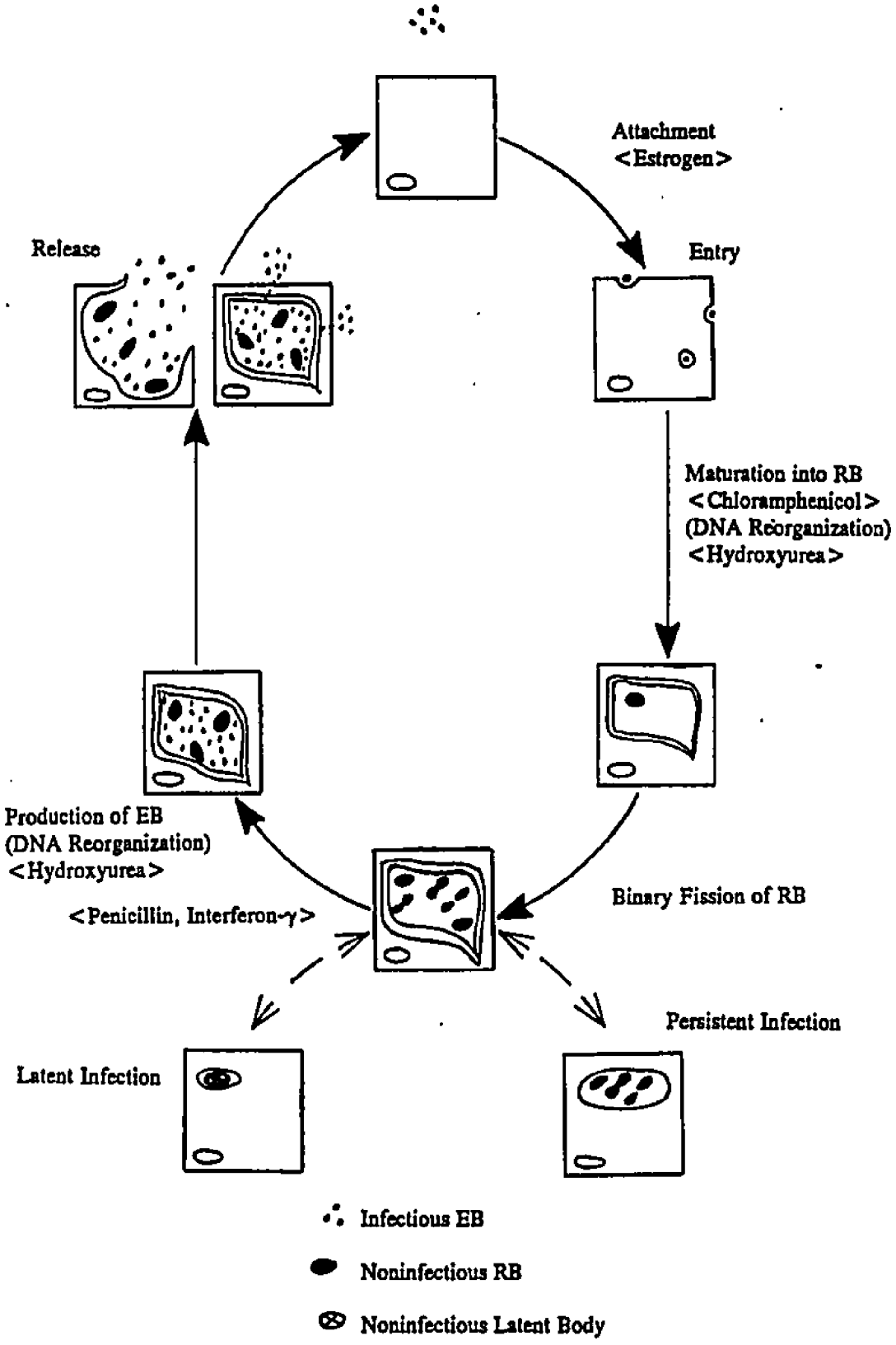
Although once thought to be a virus due to its obligate intracellular nature, subsequent analysis demonstrated that CT is a prokaryote as it contains both DNA and RNA. CT contains three species of rRNA: 21s, 16s, and 4s (Newhall 1988). This unusual bacterium has a very small genome (660 MDa) that does not code for the respiratory enzymes necessary to synthesize high energy compounds. Although glutamate, glucose, and pyruvate can be catabolized to a certain extent, this is not sufficient for survival, thus CT is entirely dependent upon the host cell for energy

(Moulder 1988). CT is classified as a gram-negative bacterium as a component of its cell wall is a lipopolysaccharide (LPS) chemically similar to enterobacterial Re LPS (Hearn and McNabb 1991). CT is unique as no peptidoglycan layer has been demonstrated nor has muramic acid been detected as a component of its cell wall, although CT does possess three penicillin binding proteins (Barbour et al. 1982, How et al. 1984). The latter distinguishes it from other bacteria which do not contain peptidoglycan, such as most Mycoplasmas.

CT has a unique biphasic developmental cycle that alternates between two morphologically distinct forms: an infectious, metabolically inert elementary body (EB) and a noninfectious, metabolically active reticulate body (RB). The EB is adapted to the extracellular environment, while the fragile RB is adapted to the intracellular environment. Each has a distinct morphology and chemical composition. The EB (400-600nm diameter) has a dense nucleus at an eccentric region and a cytoplasm that is composed of ribosomes and an amorphous material (Matsumoto 1988). The cell wall of the EB is highly disulfide cross-linked and contains at least one protein that is not found in the RB: the 60 KDa cysteine-rich outer membrane protein (Hatch 1988, Moulder 1991). The RB (size, 800-1200 nm, diameter) is surrounded by two trilaminar membranes (cell wall and cytoplasmic membranes) and tends to have a homogeneous distribution of ribosomes in the cytoplasm.

CT development consists of a multistep cycle that can be influenced at a number of steps to produce differing end results (Figure 1). Host cell ingestion of the extracellular EB is essential to CT development with attachment and entry being two

Figure 1
Developmental Cycle of CT



distinct processes (Byrne 1978). Evidence suggests that uptake is via a receptor-mediated endocytosis, possibly using host cell receptors (Hodinka et al. 1988, Wyrick et al. 1989). CT may use more than one mode of entry, dependent upon the mode of presentation to the cell, the strain of CT and the type of host cell Another important step in the chlamydial life cycle is inhibition of endolysosomal fusion to prevent degradation of the EB. It has been postulated that some strains of CT fail to grow in certain host cells due to their inability to stabilize the endolysosomal membrane and prevent degradation of the EB (Prain and Pearce 1985).

Within a few hours after attachment and entry, the EB begins to mature into the RB that is the replicative form capable of binary fission. RB metabolism relies upon the host cell for high energy compounds, vitamins, amino acids, nucleotides, and other essential nutrients. CT competes with the host cell for these nutrients such that if levels drop below a critical threshold, CT development ceases until the deprivation is reversed. As the RBs divide, the inclusion becomes filled with glycogen (18 hours post-inoculation). This glycogen is within the phagosome but is extrachlamydial. This glycogen synthesis appears to be due to a chlamydial enzyme rather than a cellular enzyme, but the purpose is unknown (Moulder 1988). The presence of glycogen in the inclusion is a major morphologic difference between CT and C. psittaci or C. pneumoniae as the latter do not sequester glycogen in the inclusion. At some point in the cycle, an unknown signal results in RB maturation into EBs. Although the assumption is that a single RB gives rise to a single EB, this has not been proven. Ultrastructural analysis has demonstrated that condensation of

the DNA may occur at several sites in the RB cytoplasm which suggests that a single RB can mature into more than one EB (Ward 1988). The regulation of the CT developmental cycle is poorly understood. Recent work has demonstrated that expression of sets of temporally distinct proteins throughout the CT developmental cycle may be regulated by a series of promoters recognized in sequence, either by cycle-dependent sigma factors or other regulatory DNA-binding proteins (Hatch et al. 1990, Moulder 1991). The maturation of RB to EB may be more complex than originally thought, possibly dependent upon signals generated in response to environmental stimuli. Cyclic nucleotides have a distinct role in CT regulation. Low levels of cyclic GMP act as a stimulator and high levels of cyclic AMP as an inhibitor of CT development (Ward and Salari 1982). The inhibitory effect of cyclic AMP is due to regulation at the transcriptional level (Kaul et al. 1990). Complex regulatory pathways appear to exist that can influence CT at many stages during development. EB maturation from the RB includes expression and incorporation of at least one EB-specific protein (60 KDa cysteine-rich outer membrane protein), extensive cross-linking of proteins in the cell wall as well as other modifications that have not yet been clearly elucidated.

Several mechanisms of CT release have been proposed. In tissue culture the predominant mechanism of release appears to be lytic disruption of the cell and subsequent dispersal of EBs (Ward 1988). In vivo, a lytic mode of release would result in tissue damage, which would explain some of the pathogenesis of the invasive chlamydiae such as LGV, but does not account for the greater majority of chronic,

asymptomatic CT infections observed. Exocytosis without concommitant cell death has been demonstrated in vitro, with EBs budding from HeLa cells (Todd and Caldwell 1985). Cell-to-cell transfer of CT also occurs either by infection of adjacent cells or transfer of the inclusion to daughter cells during cell division (Lee and Moulder 1981, Moulder 1991). CT may not be limited to a single mode of release, rather conditions in the host cell may dictate what mechanism is used. The mechanism used would influence the type of damage done to the infected tissue.

Clinical Features and Epidemiology of Genital CT Infections

CT is the most prevalent sexually-transmitted infection in the United States, with over four million new cases diagnosed annually (CDC 1985, Holmes 1981, Schachter 1978). CT infects men, women, and infants, but the consequences of such infection can be particularly serious in women. Genital infection by CT in women can result in a broad range of disease from a negligibly mild cervicitis to more serious sequelae including mucopurulent cervicitis, urethral syndrome, acute pelvic inflammatory disease (PID), salpingitis, involuntary infertility, peritonitis, and perihepatitis (CDC 1985, Lipkin et al. 1986). CT is now recognized as the leading cause of PID, resulting in 25% to 50% of the estimated 1,000,000 cases a year in the United States (Maslow et al. 1988). CT has also been implicated as the etiologic agent in a significant number of cases of ectopic pregnancy and tubal infertility (Henry-Suchet 1988, Walters et al. 1988, Quinn et al. 1987, Chow et al. 1990). Whether or not CT infection can have a deleterious effect upon pregnancy is still

debated. It has been reported that CT-infected pregnant women have a significantly higher rate of premature births and increased perinatal mortality (Martin et al. 1982), while another study had contrasting results (Alexander and Harrison 1983). CT infection has been implicated as a causative agent of spontaneous abortion, but this has not been substantiated (Quinn et al. 1987). There has been one report of fetal death from CT pneumonia that occurred across intact amniotic membranes, suggesting that CT can infect the fetus in utero, but this has not yet been definitively established (Thorp et al. 1989). The effect of CT upon pregnancy needs to be determined as the estimated prevalence of CT infection in pregnant women is 2% to 25% and is increasing. Infants delivered from an infected mother have a high incidence of inclusion conjunctivitis, pneumonia, otitis media, and gastroenteritis (Schaefer et al. 1985).

More severe complications may be the result of long-term CT infections that have gone untreated. In one study, of over 5000 women in upper East Tennessee, nearly two-thirds of those culture-positive for CT were clinically asymptomatic (Campbell and Dodson 1990). It has not been determined if all untreated asymptomatic infections will eventually progress to more serious sequelae. One of the unresolved questions in CT research is why there is such a broad range of disease states, with one women showing no apparent symptoms, while another women rapidly develops acute PID. Further work is required to determine mechanisms of CT pathogenesis and to define the factors which place a patient at greater risk for development of the more serious sequelae of CT infection. Conversely, the factors

which place a woman at risk of establishing an undetectably persistent CT infection are also unknown. Establishment of a persistent CT infection could result in reactivation at a later time, which eventually may lead to irreparable tissue damage.

Persistent CT Infections

There are many unresolved questions concerning pathogenesis of CT. There is circumstantial evidence that persistent or latent CT infections may occur, but this evidence is controversial. It has been suggested that CT infections can become latent, with reactivation at a later time by superinfection or steroid treatment (Oriel and Ridgway 1982, Yang et al. 1983). There have been documented cases of women that had been CT culture-positive subsequently becoming CT culture-negative in the absence of therapy (McCormack et al. 1979, Rahm et al. 1988). In one epidemiologic study, 25 women that had been CT culture-positive were reexamined one to two years later (McCormack et al. 1979). During this reexamination, three women who had been CT culture-negative, but CT antigen-positive, became CT culture-positive (McCormack et al. 1979). This implies that these women developed an infection undetectable by culture that later "reactivated", however reinfection has not been excluded. In a later study, a postponement in culture created a 10 week delay in treatment for 109 CT culture-positive women (Rahm et al. 1988). During those ten weeks, 17.5% of the patients developed symptoms of a genital infection and one was hospitalized for PID. It was also noted that 17 had become CT culture-negative without any treatment (Rahm et al. 1988). It has not been determined if

these women cleared the CT infection or if they developed undetectable persistent infections. In several retrospective studies, tissues from males with cystitis or prostatitis were stained with a monoclonal antibody against Chlamydia genus-specific antigen (Shurbaji et al. 1988, Shurbaji et al. 1990). The striking observation in these studies was that in a population with an average age of 60-61 years, chlamydial antigens were detected in 31-33% of the patients. This is an unusual result as prevalence of CT cervicitis is inversely related to age (Thompson and Washington 1983, Campbell and Dodson 1990).

It has not been established whether these observations reflect true latent or undetectable persistent CT infections. As culture is the "gold standard" for CT detection, a positive result using any other assay method is defined as a false positive. However, if CT can establish a persistent infection that is not detectable by culture than other assay methods may be more accurate and reflect the true state of infection in the patient. It has been noted with several nonculture techniques that some patients remain positive by several antigen-based assays, yet are CT-culture-negative (Schachter et al. 1988). This may support further the hypothesis that CT can establish persistent infections that can not be detected by culture. However, the question still remains unresolved.

One difficulty with the study of "persistent" CT infections is the definition of "latency" and "persistence". True latency, from a virology viewpoint, is a state in which the infectious agent remains in the host, but there are few signs of illness and no replication, i.e. no infectious progeny produced. The nucleic acid of the latent

agent may or may not be integrated into the host genome. A persistent infection can be defined as a chronic infection that has a low level of replication, with or without the production of infectious progeny. Even though it has been hypothesized that CT can establish "latent" infections, it is still undetermined whether or not these are true latent infections. It is possible that "latent" CT infections are actually persistent infections or active CT infections that are sequestered in the upper reproductive tract, undetectable by vaginal or cervical specimen collection.

Persistent CT infections have been demonstrated in vitro. Lee and Moulder (1981) persistently-infected McCoy cells with CT and noted that these cells underwent cycles of "wipeout", i.e. cycles of cell lysis followed by cellular regeneration. Another model of in vitro CT infection overlaid fresh cells onto CT-infected cells to demonstrate cell-to-cell transfer of CT (Benes 1990). Although, in L cells persistently infected by C. psittaci, a "cryptic form" has been described which remains hidden until some factor causes its conversion to RB, an analogous structure has not yet been found in CT persistently-infected cells (Moulder et al. 1980). The underlying mechanism(s) of CT persistence have not yet been determined, nor has the correlation of in vitro persistence to human CT infections been established.

The key to CT persistence may reside in modulators of infection. Steroid hormones (estrogen and progesterone), antibiotics (penicillin and chloramphenicol), and lymphokines (interferon-γ and tumor necrosis factor (TNF)) have been demonstrated to influence the outcome of CT infection (Byrne et al. 1989, Sarov et al. 1991). These compounds may affect the severity of the infection, the mode of

pathogenesis or the outcome, i.e. subclinical persistence vs. immune clearance. For example, women who take oral contraceptives seem to be more susceptible to CT infection (Louv et al. 1989, Kinghorn and Waugh 1981, Washington et al. 1985). Some evidence indicates that progesterone and estrogen may directly enhance the susceptibility of the cells to CT infection (Tau-Cody et al. 1988, Maslow et al. 1988). Progesterone and estrogen have been shown to affect CT infections in vivo and in vitro (Rank et al. 1982, Tuffrey et al. 1986a, Bose and Goswami 1986). Another effect of steroid hormones may be reactivation of persistent infection. In one report, cortisone-acetate treatment "reactivated" a latent CT lung infection in mice (Yang et al. 1983). The developmental cycle of CT has complex regulatory mechanisms that seem to be exquisitely sensitive to environmental stimuli.

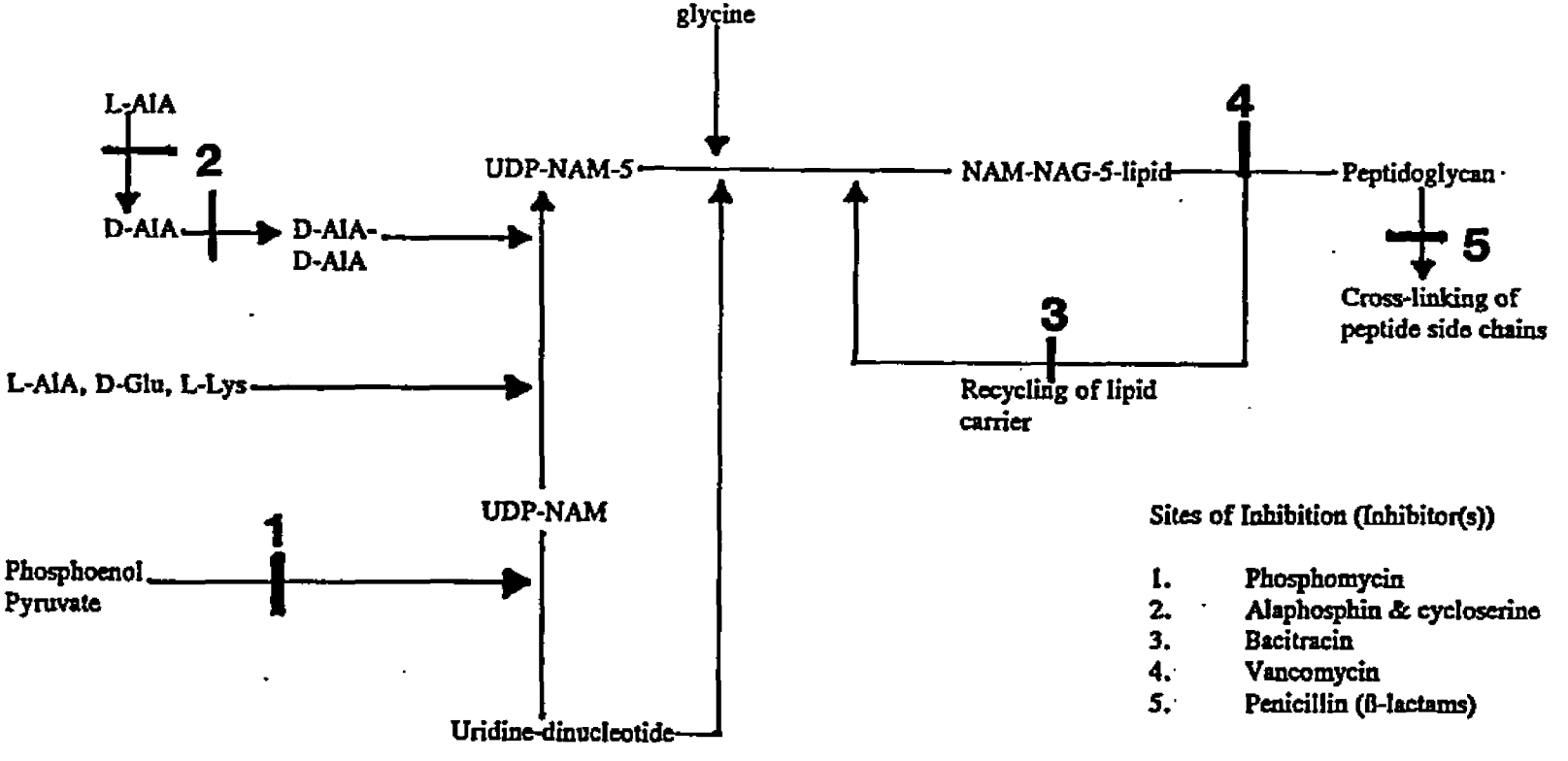
Penicillin Mechanisms of Action

The classic mechanism of action of penicillin is the disruption of peptidoglycan synthesis by competitive inhibition of the transpeptidases responsible for cross-linking the pentapeptide to adjacent glycine bridges, which result in a network of cross-linkages that give the bacterial cell wall three dimensional structure and rigidity. The peptidoglycan synthetic pathway and its inhibitors are summarized in Figure 2. It is interesting to note that the transpeptidases have acquired the label "penicillin binding proteins" as they can be isolated and their concentration determined by the uptake of radioactive penicillin.

As CT does not contain detectable peptidoglycan, it is an enigma as to how

Figure 2

Classic pathway of peptidoglycan synthesis and sites of inhibition



Sites of Inhibition (Inhibitor(s))

1. Phosphomycin
2. Alaphosphin & cycloserine
3. Bacitracin
4. Vancomycin
5. Penicillin (ß-lactams)

NAM = N-Acetyl-muramic acid
NAG = N-Acetyl-glucosamine
-5 = pentapeptide
AIA = Alanine
Glu = Glutamic Acid
Lys = Lysine

penicillin affects CT synthesis. Other bacteria without peptidoglycan and L-forms are not susceptible to penicillin (Moulder 1991). CT does contain three penicillin binding proteins (88, 61 and 36 KDa), but these have not been characterized (Barbour et al. 1982). Other antibiotics that inhibit various steps of classic cell wall synthesis have varying effects upon CT replication. Penicillin (and other β-lactams), cycloserine and bacitracin have antichlamydial activity, while alaphosphin, phosphomycin, and vancomycin exhibit no antichlamydial activity (Moulder et al. 1963, How et al. 1984). It has also been demonstrated that preincubation of CT EBs with enzymes known to cleave peptidoglycan had no adverse effect upon subsequent CT replication, further supporting the absence of a peptidoglycan layer (How et al. 1984). Early ultrastructure studies of the effect of penicillin upon C. psittaci and CT replication indicated that inhibition of EB maturation had occurred, resulting in abnormally large "RBs" (Matsumoto and Manire 1970, Kramer and Gordon 1971). Theseultrastructure studies also suggested that penicillin treatment of Chlamydia inhibits RB binary fission, although an earlier report based upon biochemical and culture data contradicts this (Tamura and Manire 1968). The observation that penicillin treatment of CT-infected McCoy cells results in abnormal inclusion formation further supports the hypothesis that penicillin inhibits maturation of the RB into the EB, but does not inhibit the initial stages of the developmental cycle (Johnson and Hobson 1977). Several mechanisms have been hypothesized to explain the unusual effect of penicillin upon CT replication. One hypothesis suggests that the classic mechanism of penicillin action is employed, however the component of the cell wall that resembles a

peptidoglycan side chain is linked to a structure other than muramic acid (Moulder 1991). A second hypothesis has suggested that abnormal forms are derived from EBs in a process analogous to spheroplast formation in other peptidoglycan-containing bacteria (Johnson and Hobson 1977). A third hypothesis has suggested that the penicillin-binding proteins found in CT have assumed new functions completely unrelated to peptidoglycan synthesis but essential to RB to EB maturation, i.e. possibly catalyzing the extensive cross-linking found in EB cell walls (Johnson and Hobson 1977). The outcome of penicillin treatment upon CT-infected cells could be very different depending upon the exact mechanism employed, i.e. eradication of the infection vs. establishment of a persistent infection. These questions have not been resolved.

Murine Model of CT Genital Infection

Development of animal model systems to study CT has been difficult since man is the only natural host, with the exception of the MoPn biovar. Although model systems of genital infection have been developed using rabbits, guinea pigs, and nonhuman primates, the mouse model seems to be the most efficient and economical model for these studies. Mice have been experimentally inoculated with human strains of CT and MoPn (Barron et al. 1984, Tuffrey et al. 1986a). Inoculation of CT into the upper genital tract of susceptible mice has been demonstrated to cause in salpingitis, as well as infertility (Tuffrey et al. 1986b, Tuffrey et al. 1990, Zana et al. 1990). The murine model of genital CT infection has been increasingly used over the

past few years, resulting in a growing body of literature concerning pathogenesis, the immune response to CT, and the effects of various antimicrobial agents upon CT replication and pathogenesis.

Although relatively poorly understood, the immune response to CT genital infection has been studied by a number of groups. Humoral immunity does not appear to play a role in protective immunity or in immune clearance of CT since reinfection can occur whether the animals were immunized or infected prior to challenge (Tuffrey et al. 1984, Ito and Lyons 1991). It has also been demonstrated that B-cell deficient mice appear to resolve genital MoPn infection as well as normal mice, further supporting the hypothesis that the humoral response is not effective against CT infection (Ramsey et al. 1988). T cells appear to be more important in CT immune clearance and have been implicated as the source of the limited immunity observed in CT infections. Nude mice infected with MoPn have been demonstrated to develop a persistent infection and this infection can be resolved by adoptive transfer of certain CT-specific T cell clones (Igietsume et al. 1991).

The immune response to CT infection appears to be protective and destructive. Although previous exposure appears to result in limited protection, challenge can result in more severe pathogenesis (Tuffrey et al. 1990). At least one CT protein (57 KDa) has been implicated in delayed hypersensitivity reactions in an ocular model of CT infection (Morrison et al. 1989). In a separate model system examining sexually-acquired reactive arthritis, mice that are sensitized to CT either by immunization or inoculation develop reactive arthritis upon intra-articular challenge

(Hough and Rank 1988, Hough and Rank 1989). The mechanisms of CT pathogenesis are still not well understood but a growing body of evidence suggests that the immune response may play an important role in tissue damage.

CT infections in mice are considered to be "self-limiting" with resolution of the infection generally determined by the absence of infectious CT in vaginal secretions (Tuffrey et al. 1984). Although persistent or latent CT infections have been hypothesized in humans, this has not been closely examined in the murine model of genital CT infection. Further work is needed to determine if persistent or latent CT infections occur in the murine model and, if so, the nature of this host-pathogen interaction.

Rationale and Goals

As the ability of CT to establish persistent infections could have a profound effect not only upon diagnosis, but also treatment, a greater understanding of the mechanisms of CT persistence and reactivation could have a significant impact upon clinical management of this infection. It was the goal of this project to develop in vitro and in vivo models necessary to examine CT persistence and reactivation at the cellular and molecular level. CT persistence in vitro was examined by using the effect of penicillin upon CT infection in McCoy cells. CT persistence and reactivation in vivo was examined using the murine model of salpingitis in C_3H mice.

CHAPTER 2

Materials and Methods

Materials

Concentrated (10x) modified Eagle's minimum essential medium (MEM), N-2-hydroxyethylpiperazine-N-2'-ethanesulfonic acid (HEPES), glutamine, fetal bovine serum (FBS), crystalline penicillin G, nystatin (10,000 U/ml), tissue culture grade penicillin/streptomycin (10,000 U/ml penicillin/10,000 μg/ml streptomycin), gentamicin sulfate (50 mg/ml), cycloheximide (CH), Dulbecco's phosphate buffered saline (DPBS), and cortisone acetate (CA) were purchased from Sigma Chemical Co. Trypsin-ethylenediaminetetraacetic acid (Trypsin-EDTA) was purchased from Gibco.

Brain heart infusion (BHI), yeast extract, tryptic soy agar, GC agar base, MacConkey agar and BHI agar were purchased from Difco. Sheep blood was purchased from Brown Laboratory. L-cysteine, vitamin K_1 and hemin were purchased from Sigma Chemical Co. Bio-x was purchased from Scott. Resazurin was purchased from Eastman Chemicals. Chopped meat carbohydrate broth (CMC) was purchased from Carr-Scarborough.

Ketaset (Aveco) and Rompun (Haver) were purchased from the Division of Laboratory Animal Resources (DLAR), James H. Quillen College of Medicine, ETSU. Depo-Provera (DP), medroxyprogesterone acetate, was generously provided by Upjohn.

Taq DNA polymerase and Eco R1 were purchased from Promega.

Immunofluorescent staining buffer (IFA buffer) was purchased from Zeus Scientific. Oligonucleotide primers for polymerase chain reaction (PCR) and an internal oligonucleotide probe were synthesized by National Biosciences. Pace 2 CT Detection System was purchased from Gen-Probe. Seakem Agarose was purchased from the Marine Colloids Division of FMC. Electrophoresis grade ethidium bromide was purchased from Fisher Biotech. DNA molecular weight markers were purchased from Bio-Rad. Polaroid T-55 film was generously donated by Sigma Chemical Co.

Cells

McCoy cells were a generous gift of Dr. J. Schachter, San Francisco, CA.

Media

McCoy cell growth medium was supplemented 1x MEM. For 500 ml growth medium, 50 ml 10x MEM was diluted with 450 ml sterile, distilled water. This was supplemented with 20 ml 27% glucose, 20 ml 1M HEPES (pH 7.3), 50 ml heat-inactivated FBS, 5 ml 7.5% sodium bicarbonate, and 5 ml 200 mM glutamine. Fresh glutamine was added every four days of storage. CT permissive medium also contained 20 μg/ml gentamicin sulfate (control medium). Nonpermissive medium contained penicillin/streptomycin at concentrations indicated in the text (penicillin medium). CH medium was McCoy cell growth medium that also contained cycloheximide (1 μg/ml). Unless otherwise indicated CH medium was supplemented with gentamicin.

BHI broth consisted of: 37 g BHI, 5 g yeast extract, 4 ml resazurin solution, 0.5 g L-cysteine hydrochloride and 10 ml vitamin K_1-hemin solution per 1000 ml broth. Vitamin K_1-hemin solution consisted of 5 ml 1.5% vitamin K_1 (w/v) in ethanol (filter sterilized) mixed into sterile 0.05% aqueous hemin (w/v). Resazurin solution was one tablet of resazurin dissolved in 44 ml water. Blood agar plates (BAP) consisted of 40 g tryptic soy agar in 1000 ml water. After steam sterilization and cooling to approximately 50°C, 60 ml sheep blood was added and the mixture was poured into petri dishes and allowed to solidify. Chocolate agar plates (CAP) consisted of two separate solutions that were steam sterilized and then mixed. In the first solution, 17 g GC agar base was dissolved in 250 ml distilled water. In the second solution, 5 g hemoglobin was dissolved in 250 ml distilled water. After sterilization, 5 ml Bio-x was added to the complete mixture and the medium was poured into petri dishes and allowed to solidify. MacConkey agar plates (MAC) were mixed according to the manufacturer's instructions.

Buffers and Solutions

Trypsin-EDTA consisted of 0.25% trypsin and 0.53mM EDTA in Hank's balanced salt solution without calcium chloride, magnesium chloride hexahydrate, or magnesium sulfate heptahydrate. Transport medium used to collect and store CT (2SP) was 0.20 M sucrose in sodium phosphate buffer (0.02 M NaH_2PO_4 and 0.02 M Na_2HPO_4) that was filter sterilized and supplemented with nystatin (25 μg/ml). IFA buffer was phosphate buffered saline mixed according to manufacturer's instructions.

DPBS consisted of 0.90 mM calcium chloride (anhydrous), 2.68 mM potassium chloride, 1.47 mM potassium phosphate monobasic, 0.49 mM magnesium chloride hexahydrate, 136.9 mM sodium chloride and 8.06 mM sodium phosphate heptahydrate (dibasic).

Tris-Borate-EDTA (TBE) was prepared as a 10x stock solution containing 0.89 M Tris-borate, 0.89 M boric acid and 0.02 M EDTA. This stock solution was diluted to the working solution by a 1:10 dilution with distilled water. Gel Loading Buffer was prepared as a 10x stock solution containing 50% glycerol and 0.25% bromophenol blue in 1x TBE buffer. Taq DNA polymerase came with a commercially prepared 10x buffer that consisted of 500 mM KCl, 100 mM Tris-HCl (pH 9.0), 15 mM $MgCl_2$, 0.1% gelatin (w/v) and 1% Triton X-100. Eco R1 came with a commercially prepared 10x buffer that consisted of 900 mM Tris-HCl (pH 7.5), 500 mM NaCl, 100 mM $MgCl_2$ and 1 mg/ml bovine serum albumin.

Monoclonal Antibodies and Stains

An FITC-conjugated monoclonal antibody specific for CT major outer membrane protein (MOMP) was purchased from Syva (Microtrak CT Culture Confirmation System). An FITC-conjugated monoclonal antibody against the genus-specific antigen LPS was purchased from Ortho Scientific (Cultureset Chlamydia Detection System).

Jones' iodine consisted of 50 ml 95% ethanol, 5 g iodine, 5 g potassium

iodide, and 50 ml distilled water, dissolved in that order and filtered through two Whatman #1 filters.

Animals

Female mice, strain C_3H/HeNCRL, were purchased from Charles River Laboratories. The mice were housed in isolation in the DLAR, James H. Quillen College of Medicine, ETSU. The mice were caged on direct bedding with each cage covered with a filter top. A twelve hour light/dark cycle was maintained, with food and water dispensed ad libitum.

Methods

Chlamydiae

The strain of CT used was isolated in our clinical laboratory from a conjunctival swab obtained from the left eye of a two-week old human female suffering from a perinatal infection. Microimmunofluorescence performed by Dr. R. Jones' laboratory (University of Indiana) determined this strain to be a serovar D. The organism has been passaged an indeterminate number of times in McCoy cells over the past five years.

The EB suspension used to inoculate McCoy cells and mice was generated by harvesting infected McCoy cell monolayers using sterile 4mm beads and vortexing each vial 15-20 seconds. The crude lysate from each vial was pooled, dispensed into

0.5 and 1.0 ml aliquots, and stored at -80°C. The lysate was titrated by inoculation of serial dilutions to determine the number of inclusion forming units (IFU) per ml.

McCoy Cell Culture

McCoy cells were grown and maintained in 150 cm^2 tissue culture flasks in control medium. Uninfected cells were grown at 37°C in a humidified atmosphere of 5% CO_2. Confluent monolayers were trypsinized, rinsed one time with control medium, and counted using a standard hemacytometer. Viability was determined by Trypan Blue dye exclusion (Mishell and Shiigi 1980).

CT Culture

The procedure used to culture infectious CT EBs was a modification of the technique of Smith (1979). Briefly, freshly trypsinized McCoy cells were diluted to a final concentration of 5 x 10^5 cells/ml in control medium and dispensed into shell vials (ashed, 1 dram vials containing 12 mm coverslips), 1 ml per vial. Each vial was closed with a sterile #0 rubber stopper and incubated at 37°C overnight. At time of inoculation, medium was aspirated from the monolayers and 100 μl inoculum added to each vial. The inoculated vials were centrifuged at 900 x g for 45 minutes, ambient temperature. After centrifugation, 1 ml/vial CH medium was added and the vials incubated at 37°C for 48-72 hours, at which time the medium was aspirated and the coverslips fixed with methanol for 10 minutes. The monolayers were stained with Jones' iodine for 30 minutes as described by Smith (1979). The stained coverslips

were inverted and mounted on a microscope slide with glycerol:Jones' iodine (1:1). The inclusions were enumerated after scanning the slide at 100x magnification. Suspected inclusions were confirmed by observation of the proper morphology at 400x magnification or by staining with FITC-conjugated anti-MOMP.

To increase the sensitivity of CT detection by culture in the in vivo experiments, blind passage was employed. Samples from vaginal swabs or tissues from the mice were inoculated in duplicate. After 48-72 hours, one replicate was stained and screened for inclusions. If the sample appeared negative, the other replicate was harvested by vortexing with glass beads, and 100 μl of the lysate was inoculated onto a fresh McCoy cell monolayer and cultured as previously described. Detection of inclusions in either the primary or the secondary culture was considered diagnostic of productive CT infection.

Quantitation of infectious CT was determined by inoculation of serial dilutions of harvested lysates using the standard CT culture technique. The number of IFU/ml was determined as follows: No. Inclusions x reciprocal dilution x 10 = IFU/ml. Yield (the number of IFU recovered per inclusion) was determined as follows: No. IFU ml^{-1} /Total No. Inclusions. Multiplicity of infection (MOI) was calculated: No. IFU Inoculated/No. Cells.

Immunofluorescent Staining of McCoy Cells

Anti-MOMP. Infected monolayers were stained using the coverslip method,

according to the manufacturers' instructions. Cells were fixed with methanol. Coverslips were then removed from the vials since staining of the coverslip in the vial did not give uniform results. For each coverslip, 30 μl of stain was layered gently on top. The slides were incubated at 37°C for 30 minutes in a moist chamber, and rinsed by vigorously dipping each coverslip into a beaker of IFA buffer 20 times. Coverslips were then blotted and mounted onto a fresh microscope slide. Inclusions were observed using a Reichert fluorescent microscope.

It was also possible to restain slides previously stained with iodine. The coverslip was placed in 1-2 ml IFA buffer and allowed to rinse at least one hour. If heavily stained with iodine, the IFA buffer was changed and the coverslip washed until all traces of the iodine were removed. Then the coverslip was stained with anti-MOMP as described above.

Anti-LPS. Infected monolayers were stained according to manufacturer's instructions. Briefly, infected monolayers were washed one time with 4ml DPBS per vial, then were washed one time with 4 ml cold (4°C) methanol per vial. Coverslips were fixed with 4 ml cold methanol for 10 minutes and airdried for 15 minutes. Dried coverslips were rehydrated by addition of 1 ml DPBS per vial and placed cell-side up onto microscope slides. Five drops of anti-LPS were added to each coverslip and the slides were incubated for 30 minutes in a moist chamber at room temperature. Coverslips were vigorously washed by dipping them into a beaker of IFA buffer and were mounted inverted on fresh microscope slides.

Effect of Penicillin Pretreatment on CT Infection

Shell vials were seeded with 1 x 10^5 McCoy cells per vial and incubated overnight at 37°C. The control medium was replaced with fresh penicillin medium (400 U/ml penicillin and 400 μg/ml streptomycin) 48 hours prior to inoculation in one group of 8 vials and at 24 hours prior to inoculation another group of 8 vials. Normal control cultures were McCoy cells grown in control medium. At the time of inoculation, the monolayers were washed twice with prewarmed DPBS and inoculated with 200 IFU CT as previously described. After centrifugation, the inoculum was removed and quadruplicate vials were fed with either control medium containing CH or CH medium containing penicillin/streptomycin (400 U/ml penicillin). The cultures were incubated for 48 hours, at which time duplicate cultures were stained with iodine or harvested to determine recovery of infectious CT. McCoy cells grown for 2 weeks in penicillin medium (400 U/ml) were used in another experiment of the same design.

Effect of Intracellular Levels of Penicillin upon CT Replication

Confluent McCoy cell monolayers in shell vials were mock-inoculated with 2SP, fed with either control CH medium or CH medium containing 100 U/ml or 400 U/ml of penicillin/streptomycin and incubated for 48 hours, 37°C. Monolayers were washed twice with DPBS (1 ml/vial) and harvested by vortexing with glass beads. Cell lysates were mixed 1:2 with freshly harvested CT and assayed using the standard CT culture. Intracellular inclusion formation was detected by iodine stain.

Penicillin Dose Curves

McCoy cells (10^5 cells/vial) were inoculated with CT either at MOI = 2.0 (2.0×10^5 IFU) or MOI = 0.0003 (3×10^2 IFU) and centrifuged for 45 minutes as described for standard CT culture. After centrifugation, inoculum was removed and each vial was fed with control CH medium or CH medium containing penicillin/streptomycin (selected concentrations of penicillin indicated in Results). Vials were processed at 52, 73, and 96 hours post-inoculation (p.i.). For each timepoint, each group was inoculated in quadruplicate; one pair of vials were stained to determine initial inclusion number and morphology, while the other pair of vials was harvested to determine recovery of infectious CT. Shell vials without McCoy cells were also inoculated in duplicate, refed control CH medium and harvested at each timepoint to determine recovery of residual inoculum (glass control).

Detection of CT rRNA

CT rRNA was detected by using the Gen-Probe PACE 2 CT detection system. This system employs a chemiluminescently-labelled cDNA probe that hybridizes to CT rRNA in a soluble hybridization assay. The sequence and specific site of hybridization are proprietary. Culture supernatants and cell lysates were diluted 1:3 in the extraction buffer provided and assayed according to manufacturer's instructions. Level of hybridization was determined by luminometer and reported as relative luminescence units (RLU).

UV-Inactivation of CT

CT suspensions were UV-inactivated using a modification of the procedure described by Maslow et al. 1988. CT inoculum (1 ml) was placed in a 60 cm petri dish and exposed to a 15w germicidal lamp at a distance of 45 cm for 10 minutes. UV-inactivated inoculum was rapidly frozen at -80°C and thawed prior to use.

Kinetics of CT Infection In Vitro

The kinetics of acute CT infection in CH-treated McCoy cells and the effect of penicillin upon such infection was examined. McCoy cell monolayers in shell vials were inoculated with 5×10^4 IFU/vial (MOI = 0.05), centrifuged at 900 x g for 45 minutes and the inoculum was removed. Some vials were fed with control CH medium and others were fed with CH medium containing 100 U/ml penicillin. At sequential times p.i., duplicate vials from each group were fixed and stained with iodine to determine initial inclusion number and morphology and a separate set of duplicate vials were harvested to determine yield of infectious CT by culture and level of CT rRNA by Pace 2 assay. Two fractions were collected from the vials that were harvested: culture supernatants were collected to determine the presence of cell-free CT, the monolayers were then washed two times with DPBS (1 ml/vial) and the cells were lysed into fresh DPBS (1 ml/vial) to determine level of cell-associated CT. Controls for detection of residual inoculum included McCoy cells inoculated with UV-inactivated CT and fed with control CH medium to determine levels of CT rRNA from a nonreplicative inoculum, and cell-free shell vials (glass control) that were

inoculated to determine levels of residual inoculum both by culture and by hybridization.

Persistent CT Infection of McCoy Cells

McCoy cells, passage number 67 from liquid nitrogen storage, were trypsinized, counted, and 3.6 x 10^6 cells were placed in each of two 50 ml centrifuge tubes. The cells were pelleted and resuspended in 1 ml CT inoculum (1.64 x 10^8 IFU/ml; MOI = 45.6) and centrifuged for 60 minutes at 900 x g, ambient temperature. The inoculum was not removed, rather the cells were gently resuspended and 17 ml McCoy cell growth medium was added, containing either gentamicin (20 μg/ml)(control medium) or penicillin/streptomycin (100 U/ml penicillin and 100 μg streptomycin)(penicillin medium). Cultures were plated in 24-well dishes at a concentration of 2 x 10^5 cells/well (1ml/well) and incubated at 37°C in a humidified atmosphere containing 5% CO_2. Three wells had sterile coverslips added to permit standard staining to observe initial inclusion formation. Every 3-5 days (up to passage number 15 and once a week thereafter) the cells were treated as follows : a.) 3 wells were fixed with methanol and stained with iodine, b.) 3 wells were washed 2 times with DPBS (1ml/well) and the cells were then scraped into 1 ml/well fresh DPBS and lysed with beads, and c.) the remaining 12 wells were rinsed as just described, trypsinized and transferred into fresh plates at a ratio of 1:3 (passage number 1-6) or 1:6 (passage number 7-22). Persistently-infected McCoy cells (CT-McCoy cells) were placed into growth medium containing different

antibiotics as indicated in figures 20 through 25 and the text in Chapter 3.

PCR

Preparation of Cell Lysates for PCR. Cell lysates from one well were extracted one time with equilibrated phenol:chloroform (Sambrook et al. 1989). One tenth volume of 3M sodium acetate was added to the aqueous phase, and then 2.5 volumes cold absolute ethanol was added to precipitate the DNA. The ethanol precipitation reactions were incubated overnight at -20°C and the DNA was pelleted by centrifuging at 11,000 x g at 4°C for 30 minutes. The pellets were airdried and resuspended in a total of 20 μl sterile water (tubes were rinsed 2 times with sterile water and the suspensions pooled).

Oligonucleotide Primers and Probe. Sequences for oligonucleotide primers and an internal probe were obtained from Holland et al. 1990: N terminal sense primer (CT1), 5'CCT GTG GGG AAT CCT GCT CAA3'; antisense orientation (CT4), 5'GTC GAA AAC AAA GTC A/TCC A/GTA GTA3'; and internal probe (CT3), 5'CCA T/CAG AAT TCC GTC GAT CAT3'. The primer pair yielded a 144 base pair (bp) fragment from a highly conserved region of the CT MOMP gene (Holland et al. 1990). The amplified fragment spanned a region containing the site for the internal probe as well as an EcoRI cleavage site which yields a diagnostic 103 bp fragment when cleaved (Holland et al. 1990).

DNA Amplification. PCR was performed on 5 μl DNA extracted from cell lysates collected from single wells. Total reaction volume was 50 μl and the final reaction mixture contained 0.125 M of each primer (unless otherwise indicated in the text); 200 μM each of dATP, dCTP, dGT,P and TTP; 1x Taq DNA polymerase buffer and 2.5 U Taq DNA polymerase. Each reaction was overlaid with 20-30 μl mineral oil. Samples underwent 40 cycles of amplification in a GTC-1 Genetic Thermal Cycler (Precision Scientific). The amplification cycle (modified from Holland et al. 1990) consisted of DNA denaturation for 1.5 minutes at 94°C, primer annealing to template DNA at 60°C for 1.5 minutes and primer extension at 72°C for 2 minutes. The positive control was high titer CT inocula and negative controls were uninfected McCoy cell lysates and water.

Optimization of PCR. Lysates from CT-infected McCoy cells were diluted to concentrations of 10, 100 and 1000 IFU/reaction. Uninfected McCoy cell lysates were mock diluted to the same levels and used as negative controls. Three primer concentrations were examined: 0.25 μM, 0.5 μM and 1.0 μM. Uninfected McCoy cell lysates were amplified only with a primer concentration of 0.5 μM. These reactions were run in a total reaction volume of 100 μl (see DNA Amplification above).

Products from like amplification reactions were pooled and precipitated with ethanol. The pelleted DNA was resuspended in 15 μl water and digested with EcoRI. Both digested and undigested samples were resolved on a 1.2% agarose gel.

EcoRI Digestion of Amplified Products

For confirmation that the proper DNA fragment was being generated , 2 μl of a positive and negative control were digested with EcoRI (14 U/reaction) in a final reaction volume of 20 μl. Samples were added to 1x EcoRI buffer and digested for 60 minutes at 37°C. Digested and undigested products were elecrophoresed on 1.2% agarose gels containing 0.5 μg/ml ethidium bromide and photographed (see Gel Electrophoresis below).

Gel Electrophoresis

1.2% agarose (w/v) was melted in 1x TBE buffer and 0.5 μg/ml ethidium bromide added. Horizontal minigels were poured and allowed to solidify. Samples were diluted 1:10 in 10x loading buffer, placed in wells and the gels were electrophoresed 40-55 minutes at 94-96 volts (Fisher Biotech). Bands were illuminated on a UV transilluminator and photographed with Polaroid Type 55 film.

In Vivo Inoculation of CT in Mice

Virgin female C_3H/HeCRL mice were divided into pretreated and nontreated groups containing infected and control animals in each group. Pretreated mice were injected subcutaneously in the back of the neck with DP in two doses: one dose (2.5 mg) seven days prior to inoculation and another dose (2.5 mg) at the time of inoculation. The mice were inoculated both intrauterinely and intravaginally with CT

using a modification of the procedure described by Tuffrey et al. (1986a). Mice were anaesthetized by intramuscular (i.m.) injection of 0.07 ml ketaset:rompun:water (1:1:1) into a hind leg. Intrauterine inoculation was accomplished by making a lateral incision over the right ovarian fat pad and injecting 0.1-0.2 ml of inoculum directly into the tip of the uterine horn using a 30g needle and a chilled syringe. The incision was sutured. The mice were then inoculated intravaginally by insertion of a blunted 23g needle into the vagina and injection of 0.05-0.10 ml inoculum. Sequential vaginal swabs were collected for the duration of the experiments and cultured with blind passage to detect infectious CT. Vaginal swabs were collected using either mini-tip culturettes (Marion Scientific) or Pur-Wraps urethral swabs (Hardwood Products Co.). Swabs were placed in 0.5 ml 2SP with 4mm sterile glass beads and vortexed vigorously 15-20 seconds prior to inoculation into McCoy cells. At sequential times p.i., mice were killed by either CO_2 asphyxiation or by cervical dislocation, gross examination was performed on the ovaries, fallopian tubes, and uterine tissues. Tissue samples of these organs were collected and saved: (a) in 0.5 ml 2SP for CT culture with blind passage to detect the presence of infectious EBs, and (b) in 10% formalin for future histologic examination and/or in situ hybridization. Tissue samples for culture were minced and vortexed with glass beads to release infectious cells. At the time of sacrifice, peritoneal swabs were obtained and cultured to determine if any post-surgical superinfection had occurred. In experiment #080190 peritoneal swabs were inoculated onto BAP, CAP, and MAC plates and into BHI broth. These media were incubated aerobically at 37°C for 48 hours to detect

presence of any aerobic growth. Swabs were also inoculated onto BAP and into CMC broth, and incubated 48 hours under anaerobic conditions to detect any anaerobic growth. In experiment #070191 peritoneal swabs were inoculated onto BAP cultured under aerobic conditions, and into CMC broth cultured under anaerobic conditions.

Experiment 1. Mice were inoculated with 9×10^5 IFU of CT (0.2 ml) intrauterinely, and with approximately 2.25×10^5 IFU of CT intravaginally (0.1ml). Controls included DP-pretreated and nontreated animals inoculated with uninfected McCoy cell lysates. The length of the experiment was 84 days p.i.

Experiment 2. All mice in this experiment were DP-pretreated with the exception of six nontreated control mice that did not undergo any type of inoculation. Control mice were DP-pretreated and nontreated mice which underwent no inoculation procedure at all. All other mice were inoculated with CT both intrauterinely (1.7×10^9 IFU (0.1 ml)) and intravaginally (8.5×10^8 IFU (0.05 ml). The length of the experiment was 22 weeks p.i. One group of CT-inoculated mice (34) were injected with 2000 U penicillin G at 9 and 16 days p.i. (2 doses of 1000 U penicillin were injected intramuscularly each day). At 10 weeks p.i., 2/3 of the mice which became culture-negative for a minimum of 2 weeks were injected with cortisone-acetate (CA) or DP subcutaneously in an attempt to reactivate the infection. CA-treated mice were

injected every other day over a period of 12 days (6 doses, 125 mg/kg per day). DP-treated mice were injected with 2.5 mg DP once a week for 2 weeks. CT-inoculated mice, from the same subpopulation, injected with saline (following the CA schedule), served as control animals. The first reactivation group of mice was killed 15-16 weeks p.i. At 17 weeks p.i., all other mice (culture-negative for a minimum of 2 weeks) were subjected to the same steroid induction regimen and were killed at 22 weeks p.i.

CHAPTER 3

Results

In Vitro Models

Effect of Penicillin on CT Inclusion Morphology

Penicillin treatment of CT-infected McCoy cells dramatically altered inclusion morphology even at concentrations considered subinhibitory (10 U/ml). When stained with iodine, penicillin-treated CT-inoculated McCoy cells did not have any normal inclusions but did have large vacuoles that appeared to contain little or no glycogen (Figure 3A-B). Staining these monolayers with a monoclonal antibody (MAb) against CT MOMP demonstrated that this chlamydial antigen was present in these abnormal inclusions, although the staining was less intense and the antigen was deposited differently than in normal inclusions (Figure 4A-B). The deposition of MOMP in these abnormal inclusions resembled large open rings or "bubbles" (Figure 4B). The discrete, highly fluorescent particles (elementary bodies) observed in normal inclusions at this time p.i. were almost completely absent in the abnormal inclusions. Staining of the penicillin-treated, CT-inoculated McCoy cells with a MAb against chlamydial LPS yielded a similar result. Chlamydial LPS was present in these abnormal inclusions and was deposited in a similar fashion, i.e. "bubbles".

Figure 3

CT-inoculated McCoy cells stained with iodine (40x).

A. Untreated cultures with normal inclusions.

B. Penicillin-treated cultures with abnormal inclusions and vacuoles (some indicated by arrows).

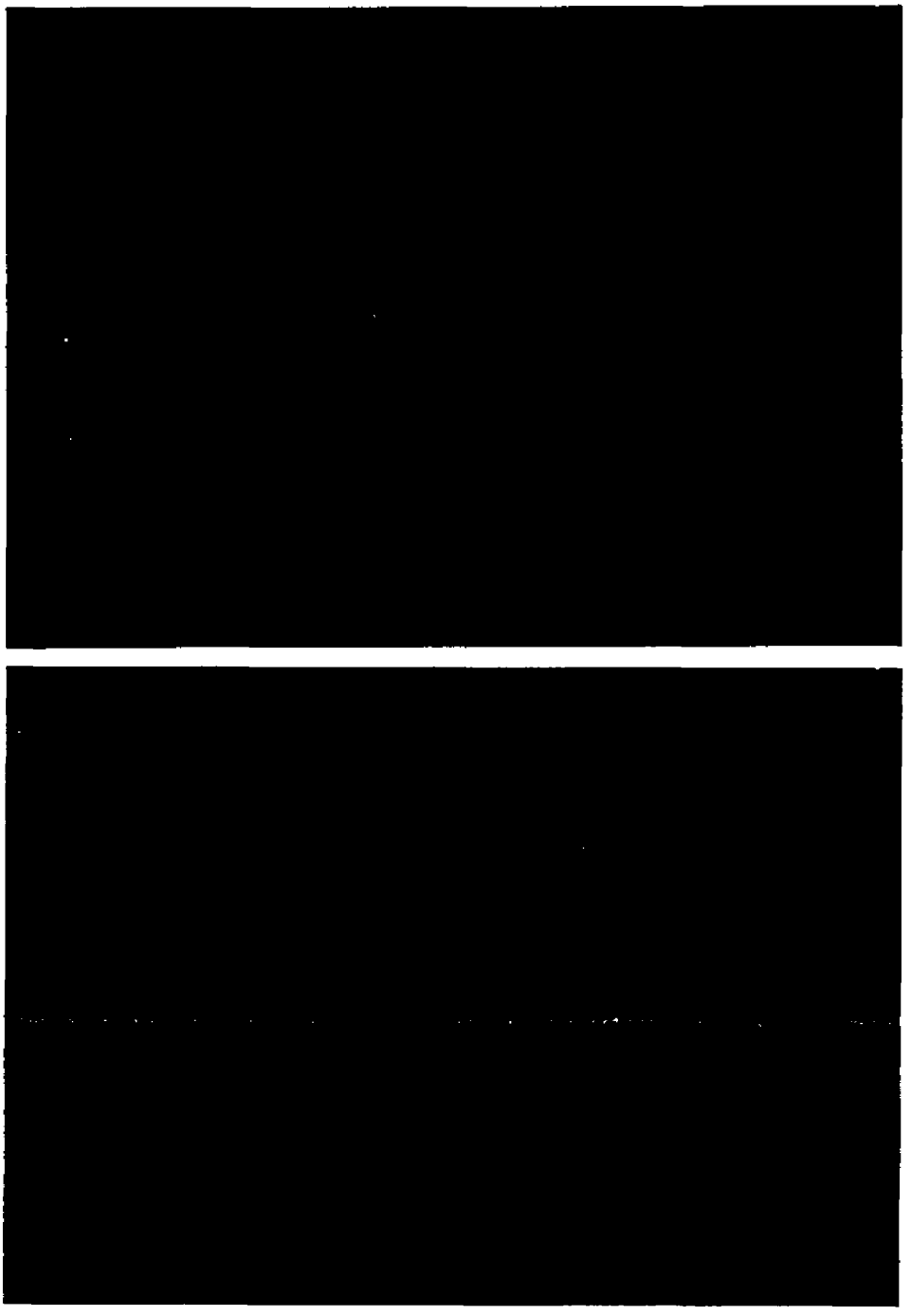
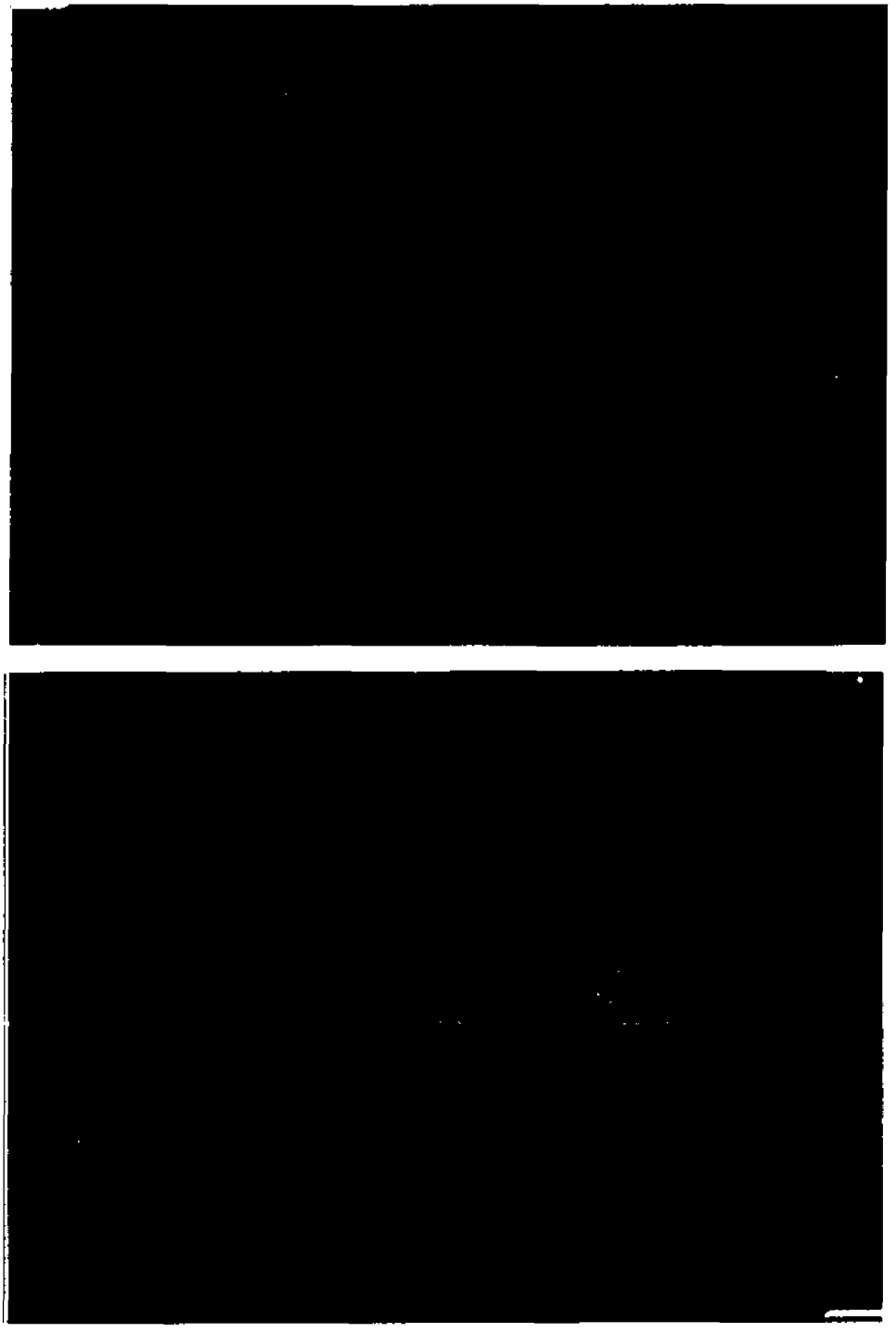


Figure 4

CT-inoculated McCoy cells stained with anti-MOMP (100x).

A. Untreated cultures with normal inclusions.

B. Penicillin-treated cultures with abnormal inclusions.



Penicillin Pretreatment of McCoy Cells

Penicillin pretreatment of McCoy cells did not affect subsequent CT replication provided that the penicillin was removed and monolayers were washed prior to inoculation. Neither initial inclusion formation (number and morphology) nor yield of infectious CT were altered by penicillin pretreatment of the monolayers (Figure 5). Even cells grown for two weeks in medium containing 400 U/ml penicillin supported CT replication provided that the monolayers were washed prior to inoculation (Figure 6). Intracellular levels of penicillin did not appear to inhibit CT replication as lysates from uninfected McCoy cells grown in 100 or 400 U/ml penicillin (washed prior to lysis) did not inhibit inclusion formation, as compared to control cultures (Figure 7).

Penicillin Dose Curves

In order to determine if the effect of penicillin upon CT infection was dose dependent, a range of penicillin concentrations were tested and compared to control cultures. The parameters examined were initial inclusion formation and yield of infectious CT. The penicillin concentrations examined were 10 U/ml, 50 U/ml, 100 U/ml and 200 U/ml. These concentrations bracketed a reported MIC value of 16 to >64 U (Lee et al. 1978). When stained with anti-MOMP, the number of abnormal inclusions in the penicillin-treated CT cultures did not differ substantially from the number of normal inclusions found in cultures grown in CT-permissive medium (Figure 8). A similar result was observed when penicillin-treated CT cultures were stained with anti-LPS (Figure 9). Comparable numbers of inclusions were detected using anti-MOMP or

Figure 5

Effect of penicillin pretreatment of McCoy cells upon subsequent CT replication. 0H, 24H and 48H represent the hours of penicillin pretreatment prior to CT inoculation. C represents control medium placed on the cultures after inoculation. P represents penicillin medium placed on the cultures after inoculation.

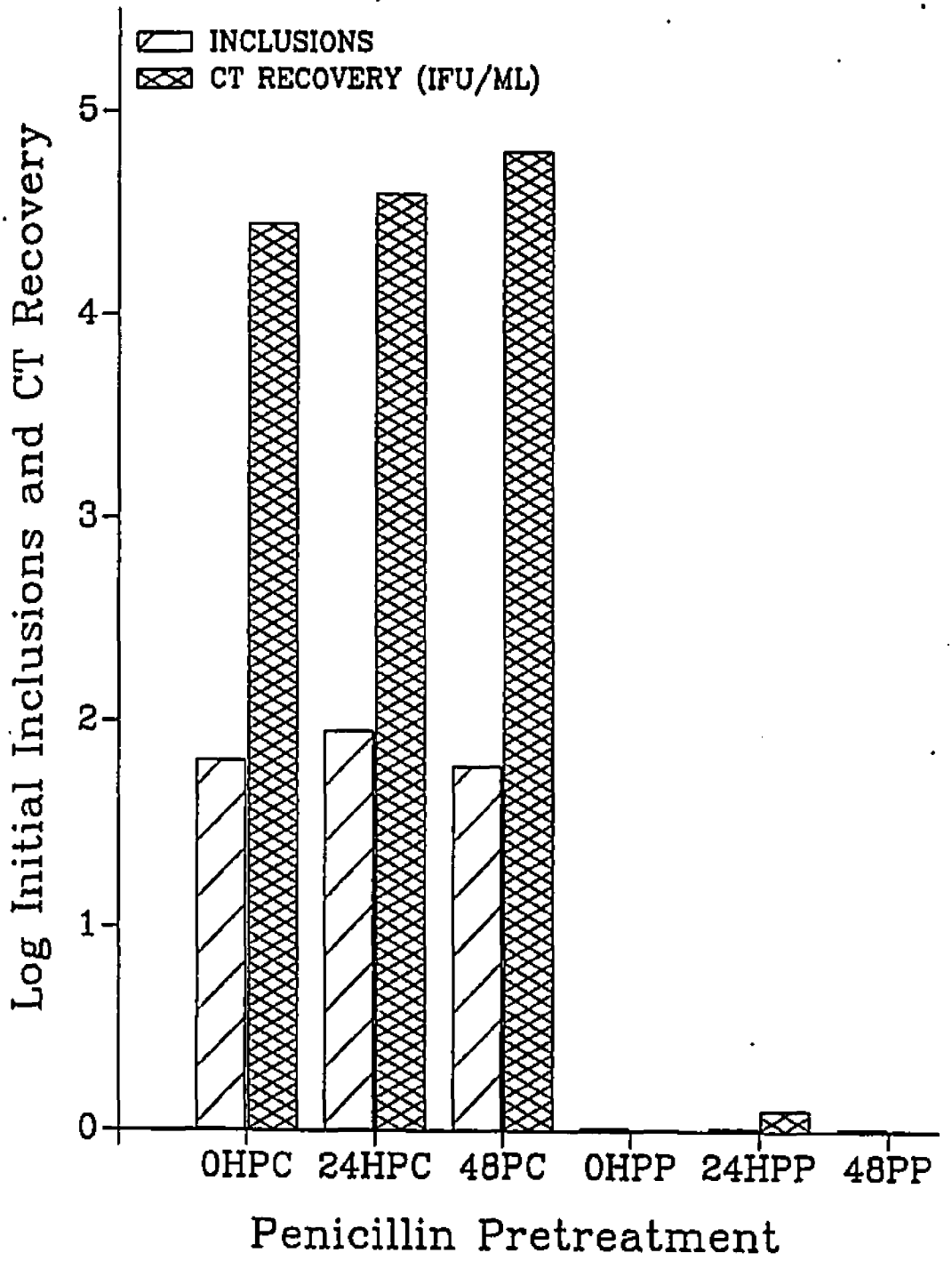


Figure 6

Effect of penicillin pretreatment of McCoy cells for two weeks prior to inoculation upon subsequent CT replication.

CCC = Control culture

PPC = Penicillin pretreatment for 2 weeks; control medium p.i.

PCC = Penicillin pretreatment for 2 weeks; control medium -48 hours; control medium p.i.

CCP = Control medium; penicillin medium p.i.

PPP = Penicillin pretreatment for 2 weeks; penicillin medium p.i.

PCP = Penicillin pretreatment for 2 weeks; control -48 hours; penicillin medium p.i.

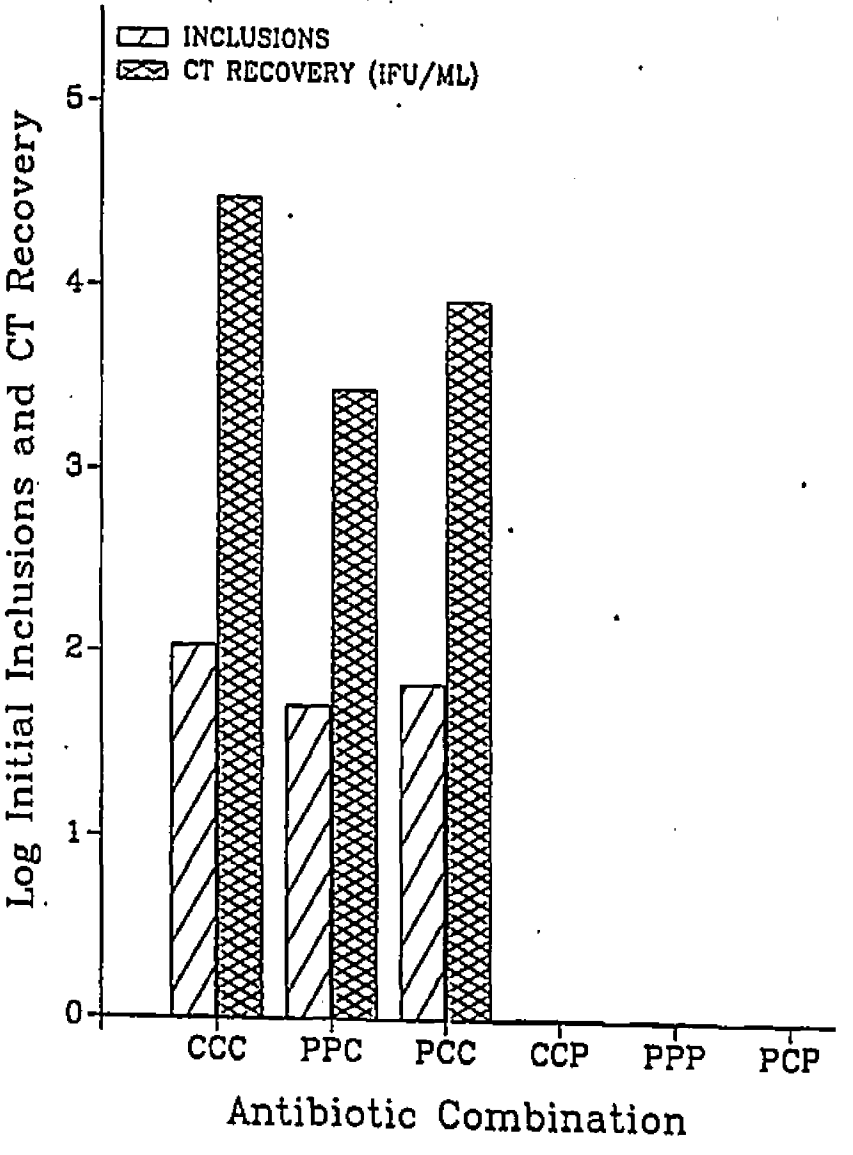


Figure 7
Effect of intracellular levels of penicillin upon CT replication.

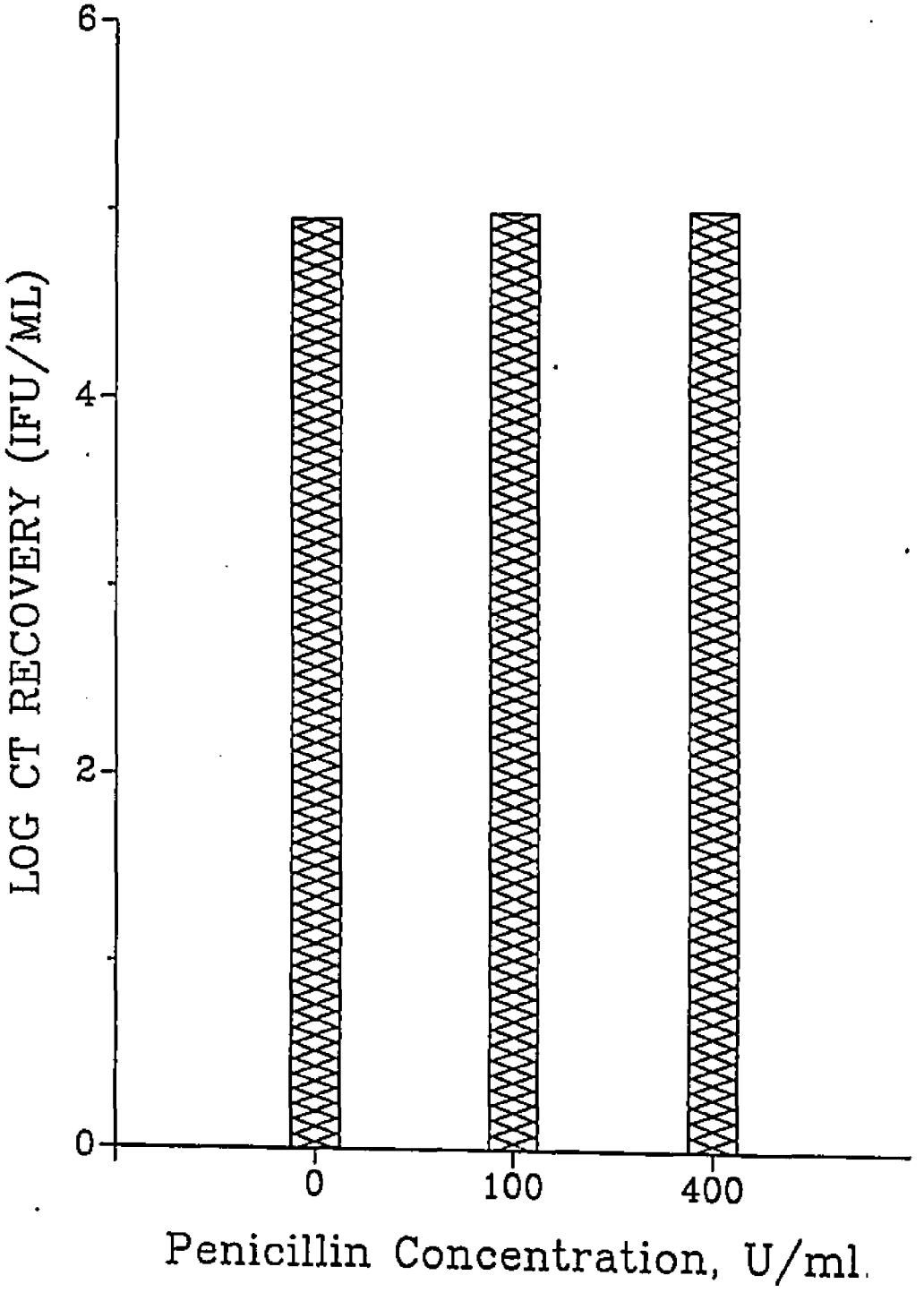


Figure 8

Effect of different doses of penicillin upon intracellular inclusion formation in CT-infected McCoy cells stained with anti-MOMP.

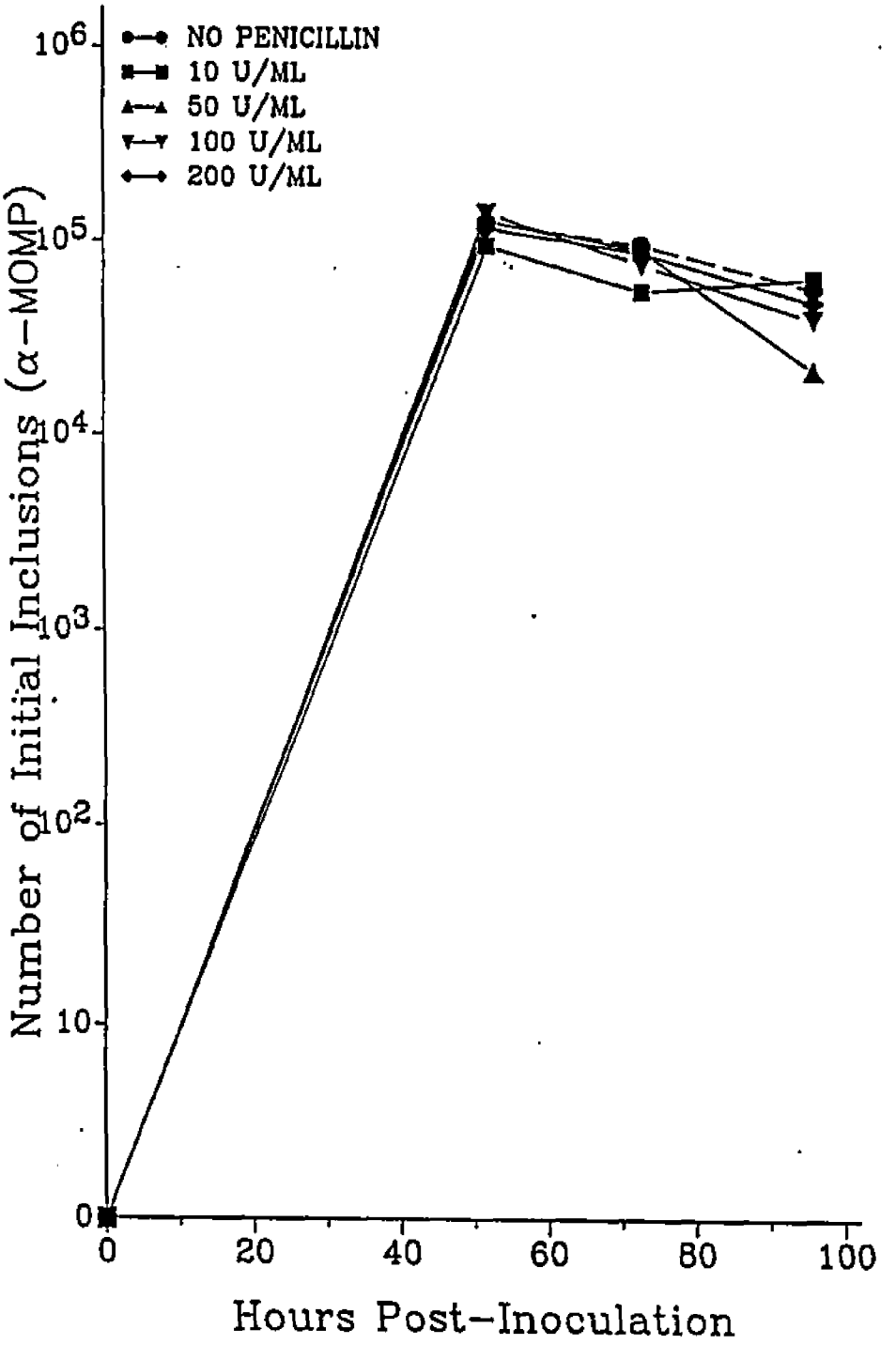
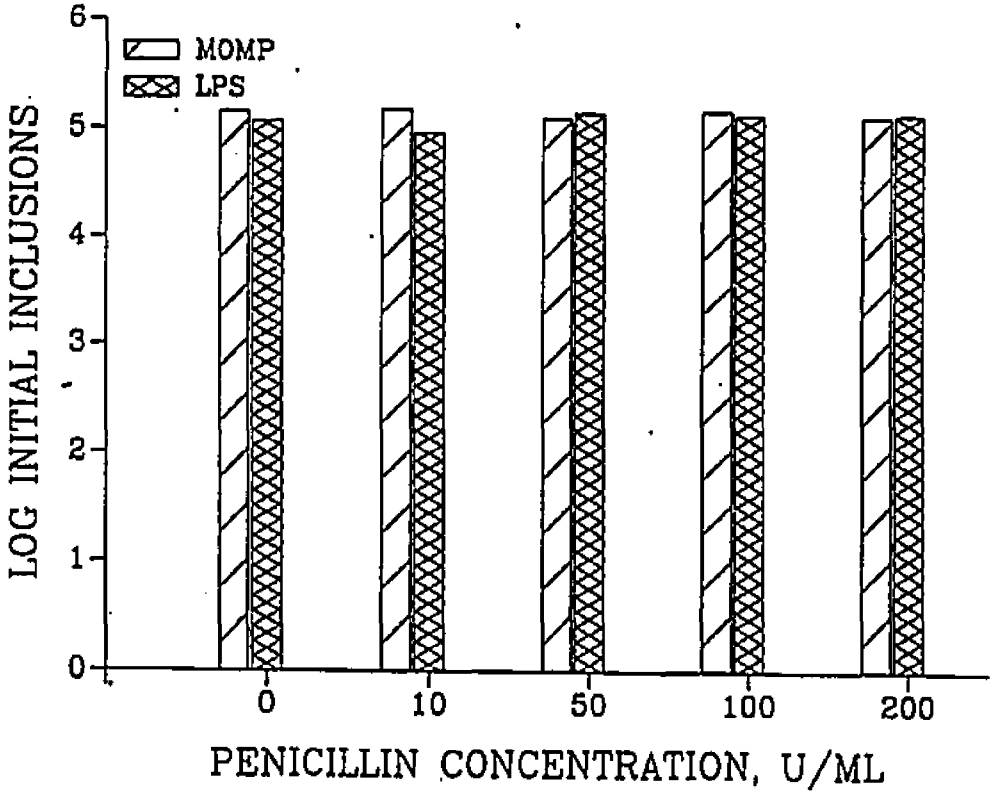


Figure 9

Effect of different doses of penicillin upon intracellular inclusion formation in CT-infected McCoy cells stained with anti-LPS.



anti-LPS (Figure 9). This result was observed over the range of penicillin concentrations tested. Initial inclusion formation was not dose dependent over the range tested since similar results were seen whether 10 U/ml or 200 U/ml penicillin were added. Addition of penicillin at the time of inoculation did not inhibit initial inclusion formation. However, addition of penicillin to CT cultures did drastically reduce the yield of infectious CT from these cells. When an MOI of 2.0 was used, the number of IFU recovered from penicillin-treated CT cultures was reduced greater than 1,000,000-fold (Figure 10). This reduction was observed over the range of penicillin concentrations tested and was not dose-dependent (Figure 11). When an MOI of 0.0003 was used, no IFU were recovered from penicillin-treated CT cultures at any penicillin concentration tested, although abnormal initial inclusions were observed (Table 1 and Figure 12). With a sufficiently large inoculum (i.e. high MOI), penicillin did not completely inhibit CT replication, although infectious elementary body production was dramatically reduced.

Effect of Penicillin upon the Kinetics of Acute CT Infection

Although little or no infectious CT was produced in penicillin-treated cultures, it remained possible that noninfectious reticulate bodies or defective elementary bodies were present. Culture would not detect either of these forms. A cDNA probe specific for CT rRNA was employed to detect CT in tissue culture supernatants or cell lysates. A standard curve comparing IFU to RLU was performed using the probe assay (Figure 13). Linear regression analysis showed that 1 IFU corresponded to 53 RLU. However, this value was equal to or less than the nonspecific background detected when negative

Figure 10
Effect of penicillin upon the yield of infectious CT (MOI = 2.0).

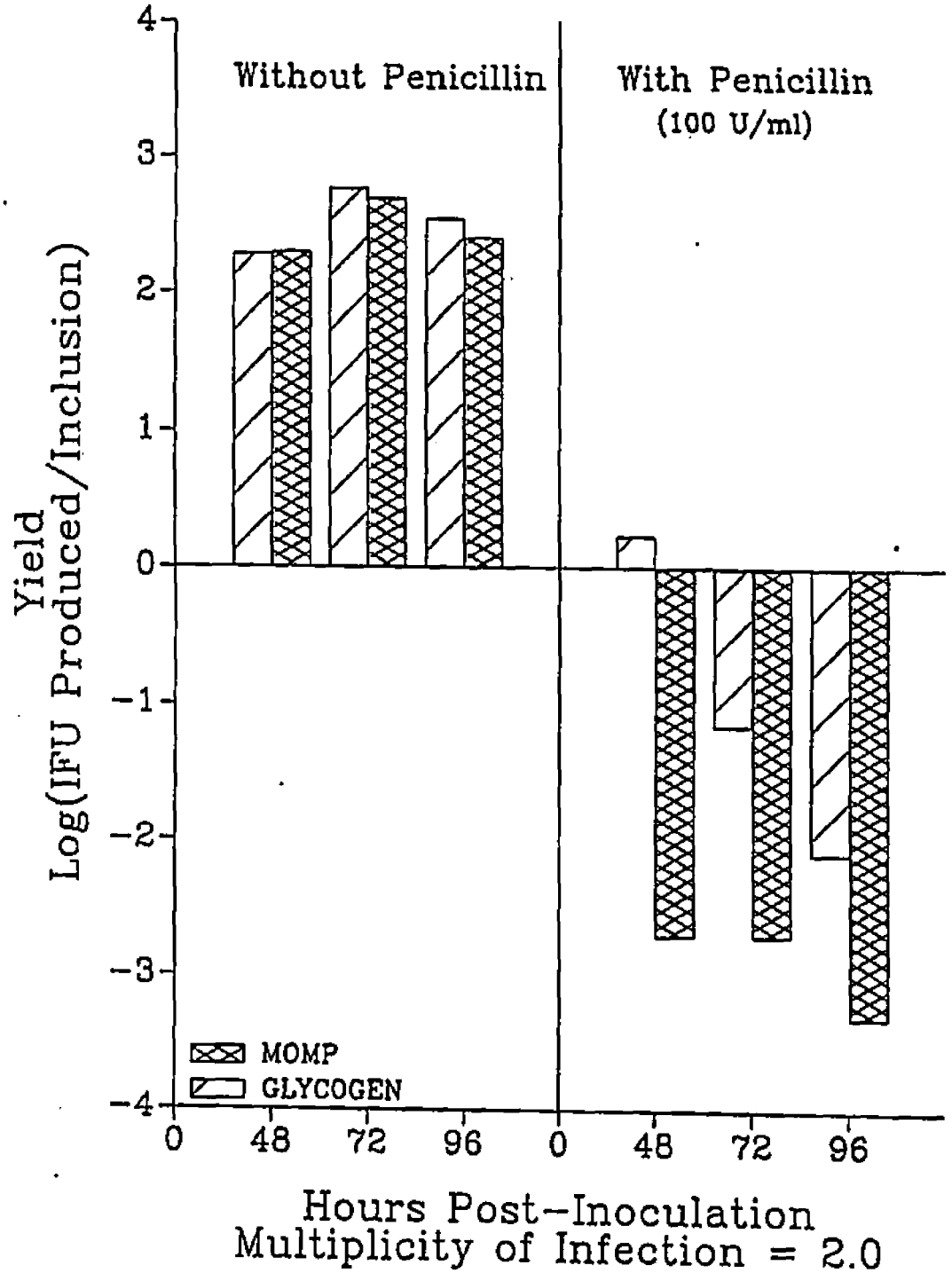


Figure 11
Effect of penicillin upon recovery of infectious CT (MOI = 2.0).

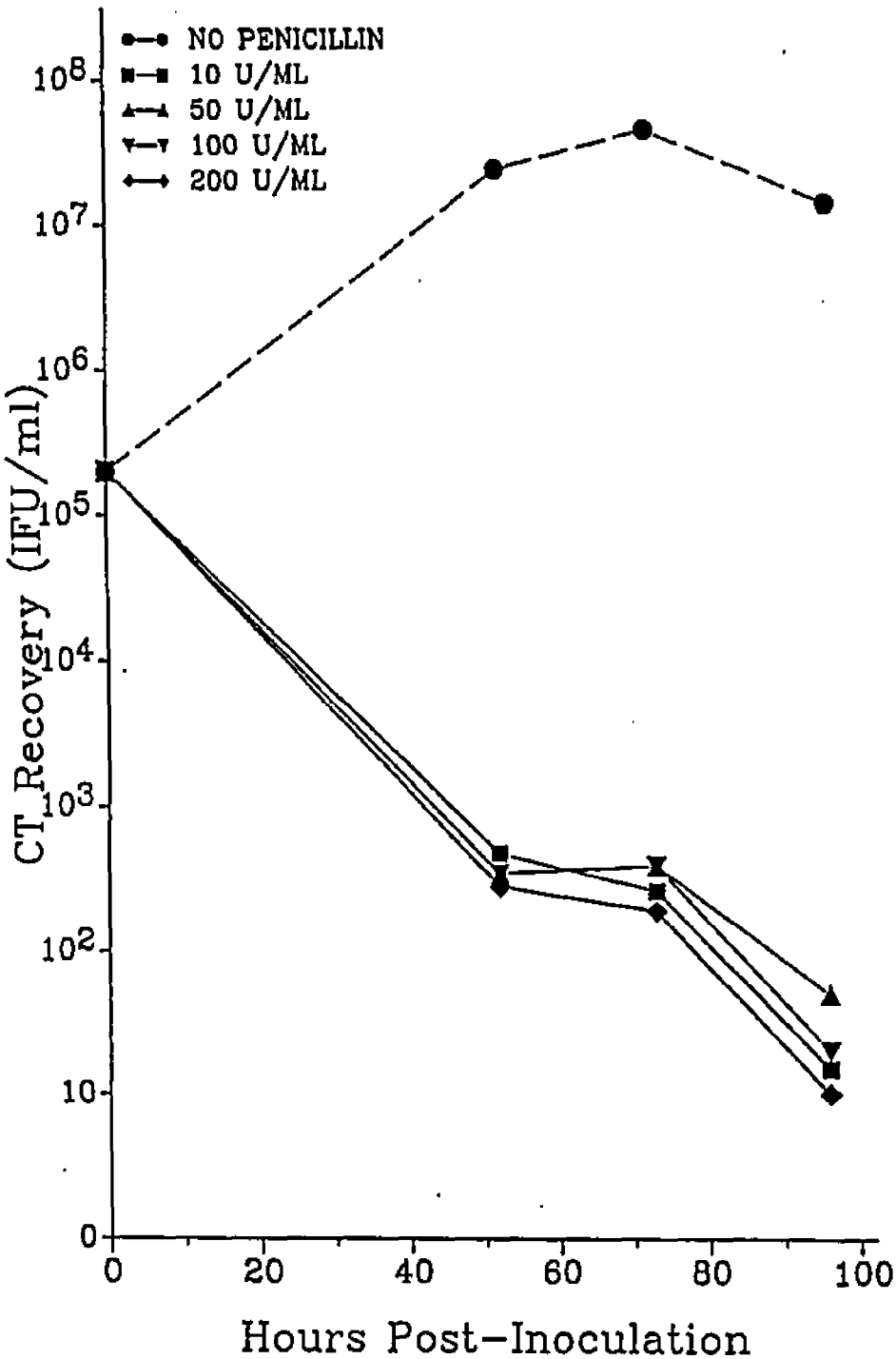


Table 1 Intracellular Inclusion Formation and CT Recovery from McCoy Cells Inoculated with CT in Varying Penicillin Concentrations (MOI = 0.0003)

Dose Penicillin (U/ml)	Time p.i. (h)	Initial Inclusions [A]				Recovery [A] (IFU/ml)
		No. I_2	Type [B]	No. MOMP	Type	
0	52	258	N	214	N	550
10		0.5	S	75	I	0
50		0		76	I	0
100		0		92	I	0
200		0		52	I	0
0	73	200	N	155	N	1.3×10^4
10		0.5	S	72	I	0
50				34	I	0
100				74	I	0
200				47	I	0
0	96	94	N	202*	N	1.1×10^5
10		0		14*	I	0
50		0		27	I	0
100		0		37	I	0
200		0		28	I	0

[A] All values are $\bar{x}$, except where marked *

[B] Inclusion morphology: N = Normal; S = Small but complete; I = Incomplete

Figure 12
Effect of penicillin upon the yield of infectious CT (MOI = 0.0003).

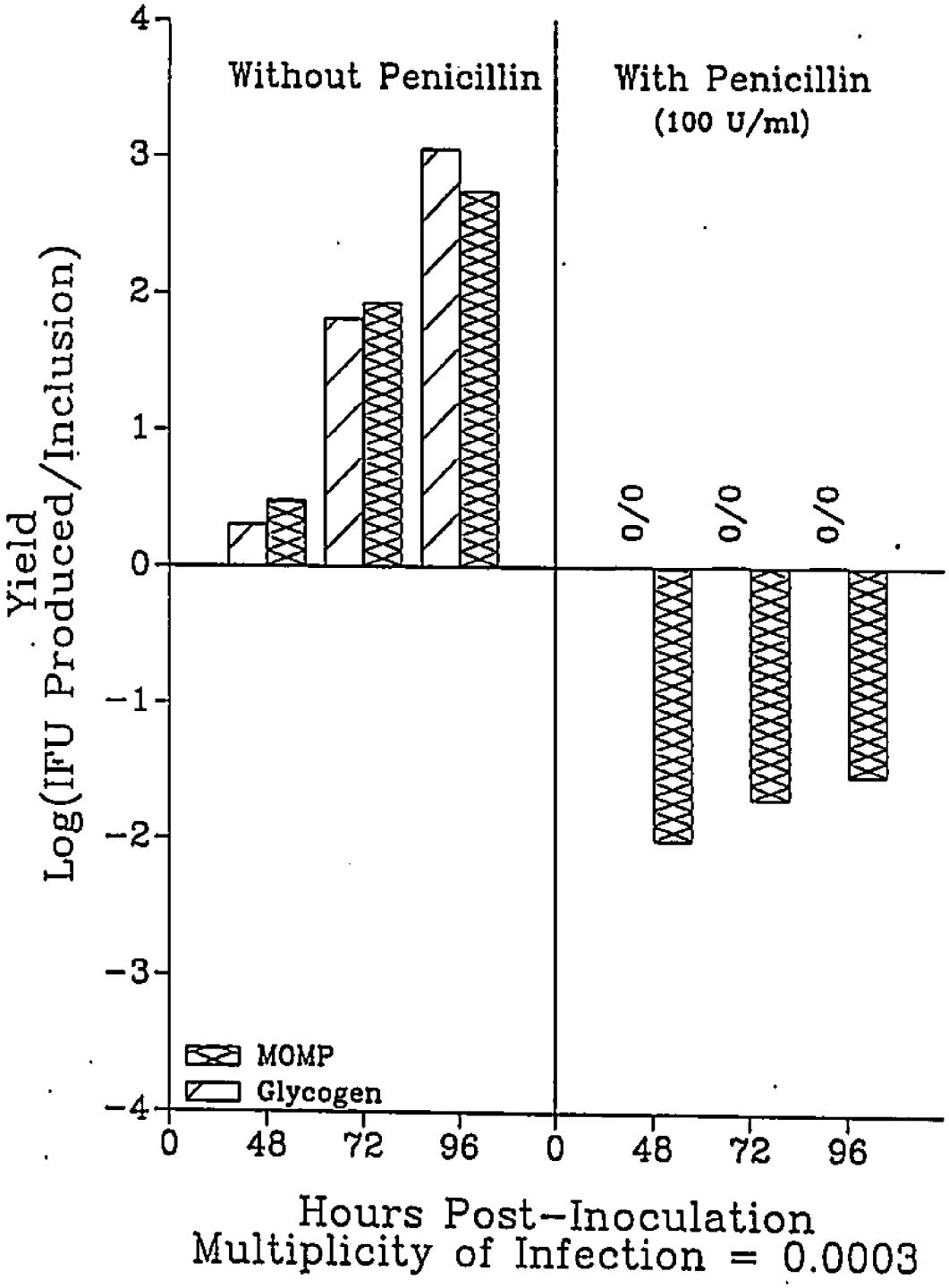
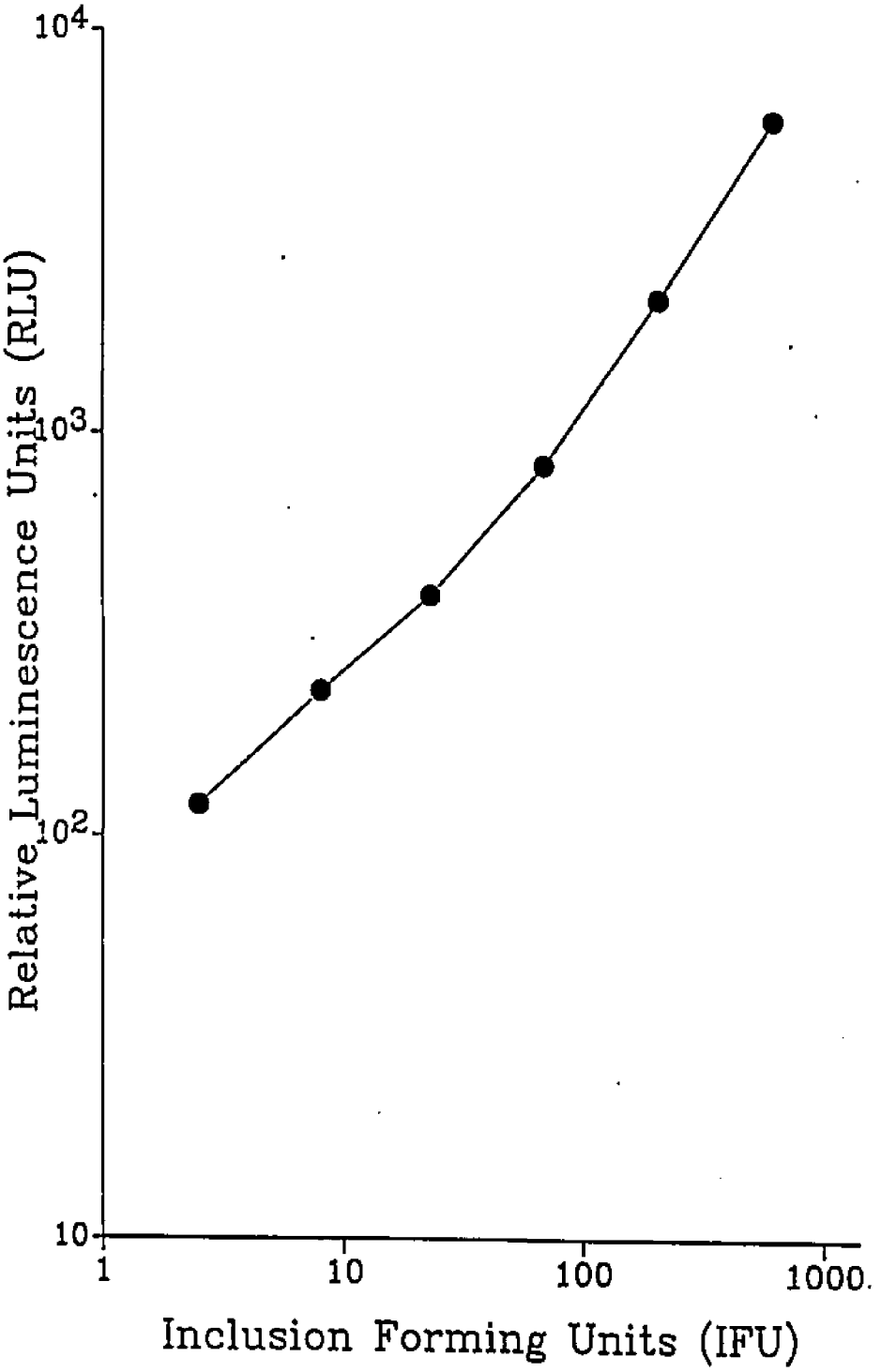


Figure 13
Standard curve comparing inclusion forming units to relative luminescence units.



control nucleic acid was assayed. Therefore, the assay cutoff value chosen was 1.5 times the mean of the triplicate negative controls and was consistently greater than the highest of the negatives. The probe assay was very sensitive when thus employed and detected as few as 5 IFU (Figure 13). The probe assay also could detect UV-inactivated CT with the same sensitivity (Figure 14). The probe assay was more sensitive than culture as CT does not need to be viable or infectious to be detected by the former. Reticulate bodies and nonviable or defective elementary bodies could be detected using this technique. A kinetic study of acute CT infection in CH-treated McCoy cells was performed which employed both culture and probe assay. Initial inclusion formation was as observed previously. Residual inoculum was not detectable by culture in either fraction of the glass control by 15 hours p.i. Infectious CT was not recovered from any culture inoculated with UV-inactivated CT. CT culture of supernatants from penicillin-treated cultures was not performed as the penicillin in the medium would interfere with accurate quantitation of infectious CT. Recovery of IFU from supernatants of untreated cultures exhibited a typical growth curve with the minimum at 15 hours p.i. (305 IFU), and rapidly increasing to a maximum number of 7.1×10^5 IFU/ml at 73 hours p.i. (Figure 15A). A similar growth curve was observed when cell lysates from untreated cultures were assayed for infectious CT (Figure 15B). The growth curve obtained from the cell lysates of penicillin-treated CT cultures was quite different from that of the control with a single peak of IFU produced at 32 hours p.i. (80 IFU/ml). Recovery gradually decreased until no IFU were recovered at 98 hours p.i. (Figure 15B). It was not possible to determine if this small peak of IFU production reflected abortive replication or that

Figure 14 .
Comparison of standard curves generated by using viable and UV-inactivated CT.

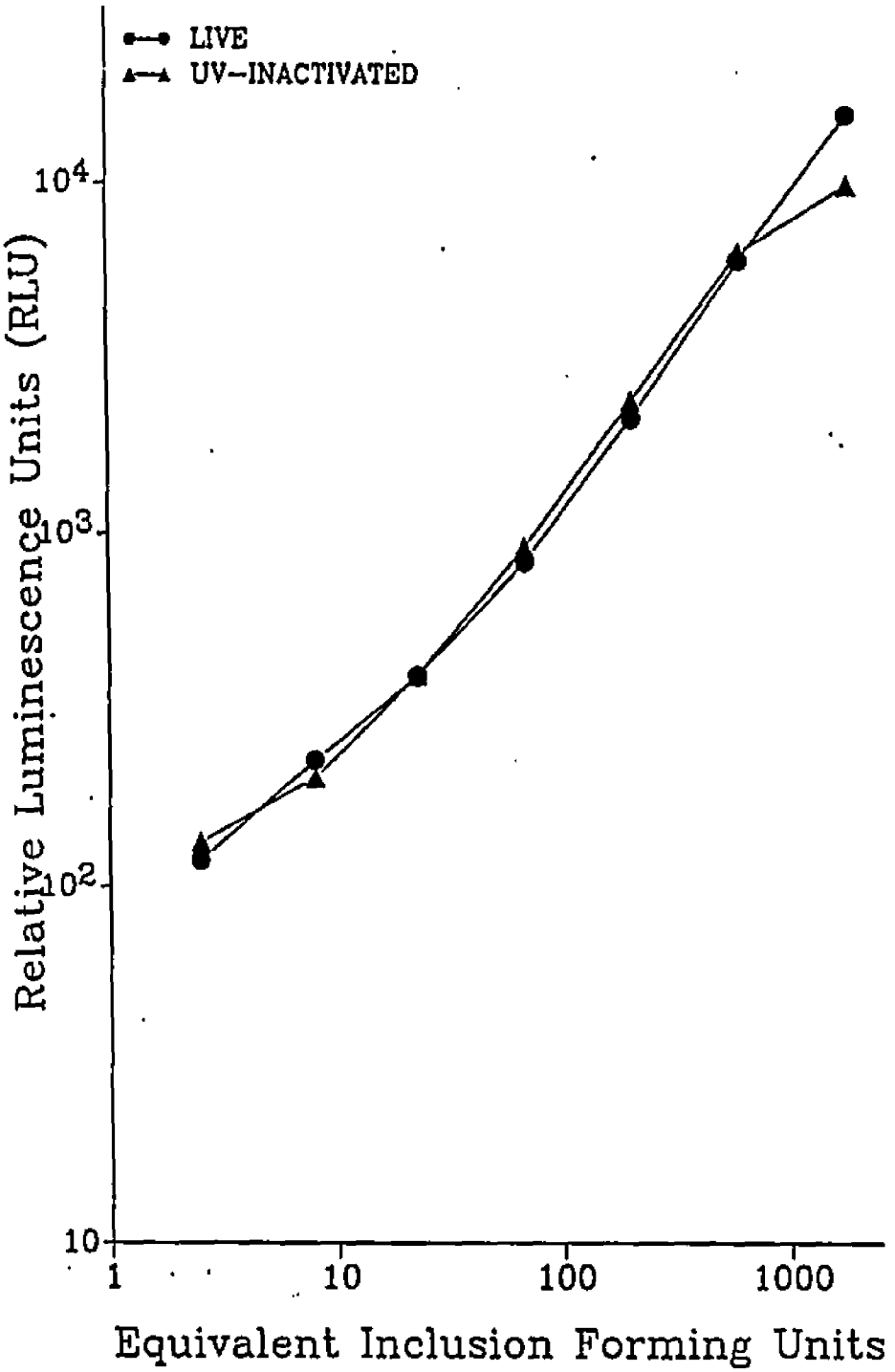
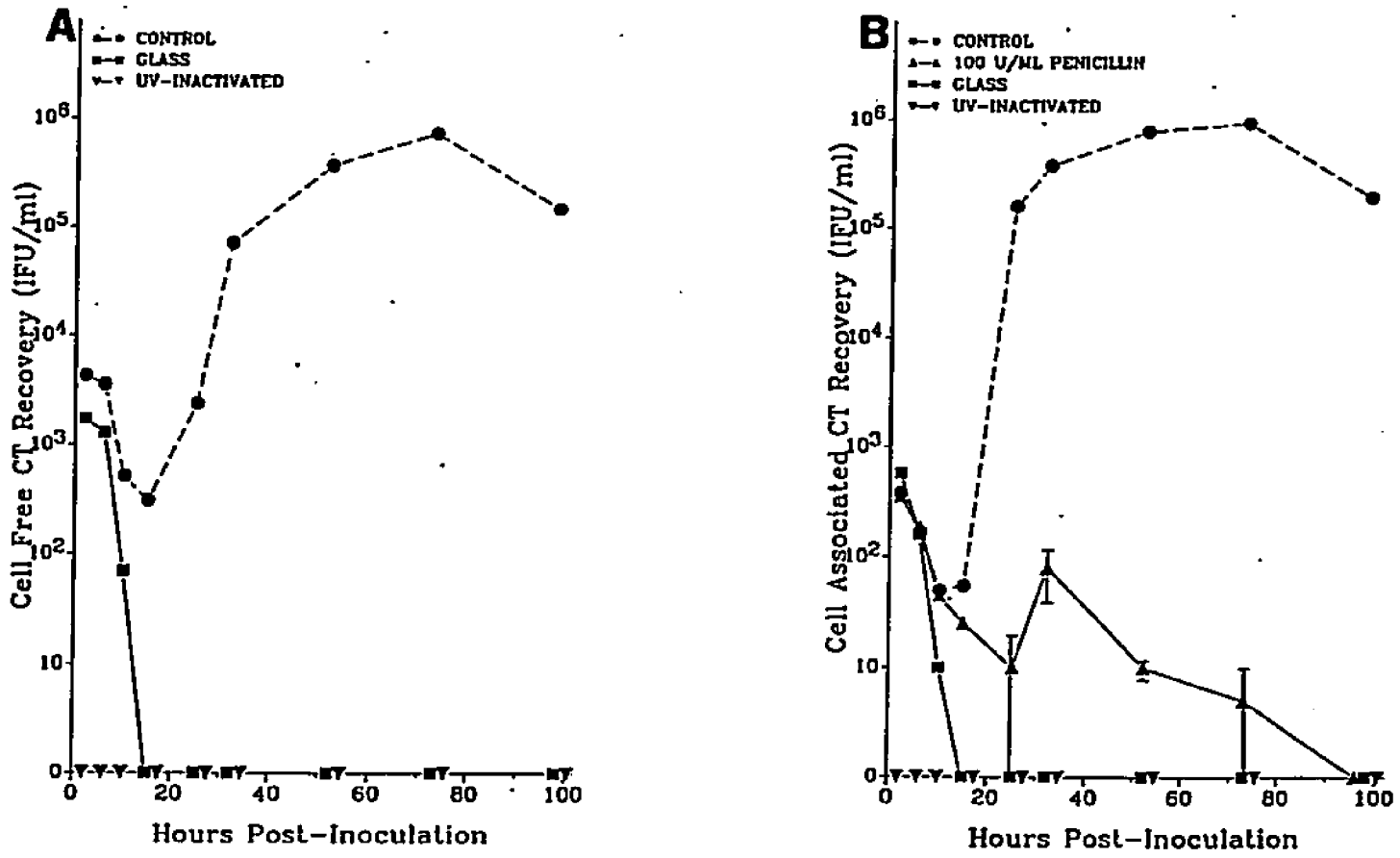


Figure 15

Kinetics of the recovery of infectious CT from CT-inoculated McCoy cells in the presence and absence of penicillin (100 U/ml).

A. Cell-free IFU.

B. Cell-associated IFU.



CT did not become inactivated as quickly in the presence of cells, i.e. residual inoculum.

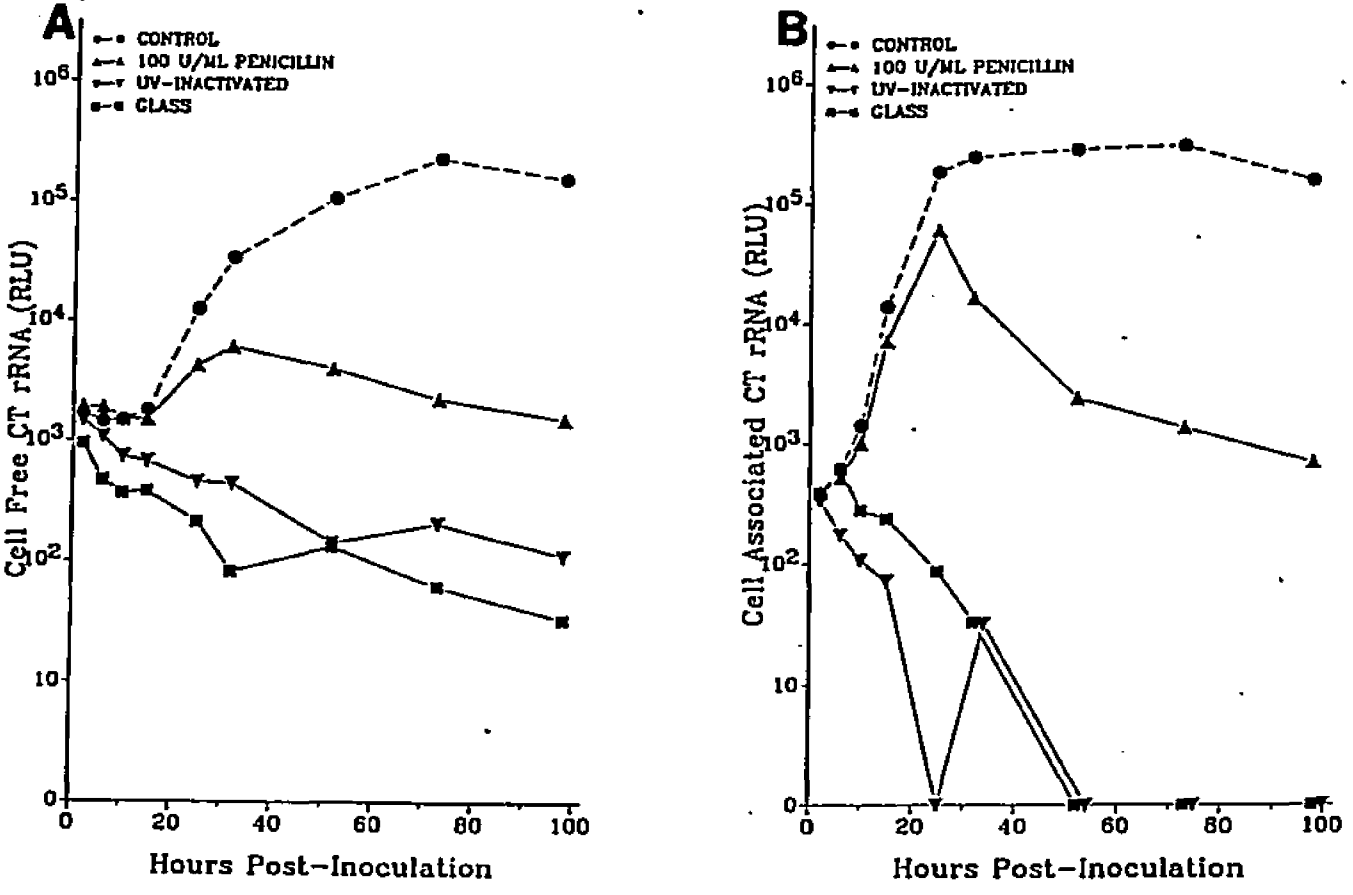
Both culture supernatants and cell lysates from control and penicillin-treated CT cultures were assayed by DNA-rRNA hybridization (Figure 16). CT rRNA was detected in the supernatants of control cultures in increasing amounts from 25 hours p.i. onward (Figure 16A). This rRNA reflected release of CT elementary bodies into the supernatant and/or release of reticulate bodies into the medium due to cell lysis. CT rRNA was detected in the supernatants of penicillin-treated CT cultures throughout the course of the experiment although at lower concentrations than observed in untreated CT-infected cultures (Figure 16A). There was a peak of hybridization at 32 hours p.i. (6,307 RLU) gradually declining by 98 hours p.i. to a value of 1,684 RLU which was still unequivocally positive (Figure 16A). Both CT-inoculated cell-free shell vials and McCoy cells inoculated with UV-inactivated CT were positive by probe assay throughout the time range examined, but these controls showed no evidence of CT replication as the values remained the same or decreased over time (Figure 16A). These controls were included as a measurement of CT rRNA in residual inoculum. The penicillin-treated CT cultures produced levels of hybridization far greater than these controls yet significantly less than the levels found from supernatants of CT-infected control normal cultures (Figure 16A). As with CT rRNA detected in the supernatants of control cultures the CT rRNA from the penicillin-treated cultures could represent CT elementary bodies released into the culture fluids and/or reticulate bodies from lysed cells. Since culture was not performed on the supernatants from penicillin-treated cultures, it was not possible to determine if the CT released into the supernatant was infectious. Defective elementary

Figure 16

Kinetics of the levels of CT rRNA in CT-inoculated McCoy cells in the presence and absence of penicillin (100 U/ml).

A. Cell-free RLU.

B. Cell-associated RLU.



body production was neither proved nor disproved by these results.

Cell-associated CT rRNA was detected by hybridization in lysates of untreated and penicillin-treated CT cell cultures (Figure 16B). The levels of CT rRNA detected in untreated and penicillin-treated cultures were essentially the same during the first 10 hours p.i. (Figure 16B). By 15 hours p.i., the level of CT rRNA in penicillin-treated cultures was only half that detected in untreated CT-infected control cultures (Figure 16B). This divergence continued until the level of CT rRNA from penicillin-treated cultures peaked at 25 hours p.i. and then declined rapidly (compared to the level found in untreated CT cultures). However, the level remained significantly higher than the levels of CT rRNA observed in UV-inactivated CT cultures and the glass control.

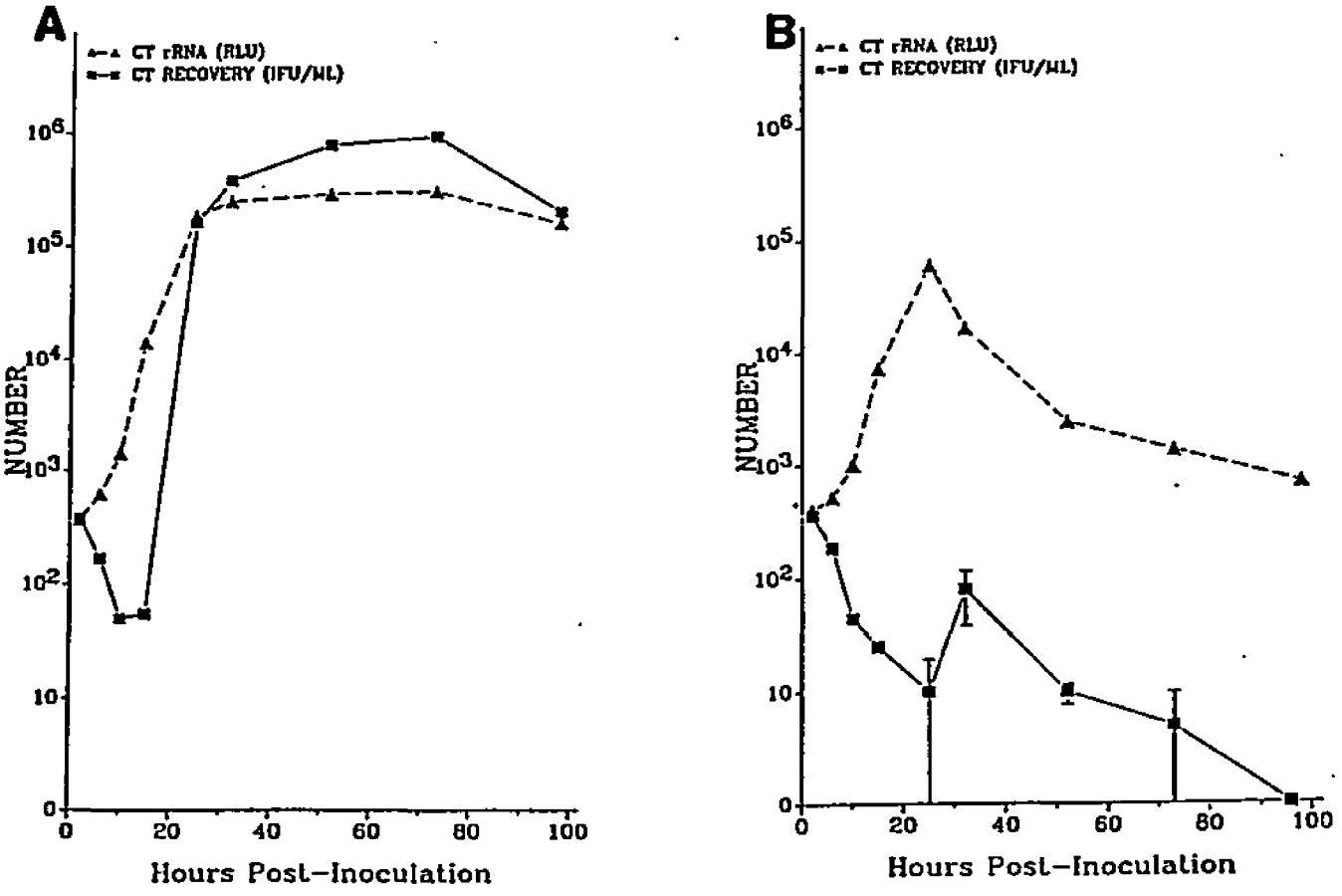
Cell-associated CT recovery from untreated cultures showed the expected decrease (eclipse) in early hours of infection as infectious elementary bodies matured into noninfectious reticulate bodies (Figure 17A). That this decrease in infectivity reflected maturation of EB into RB was supported further by the dramatic increase in the amount of CT rRNA detected by probe assay, particularly between 2 and 25 hours p.i. The rapid expansion of the CT rRNA pool was followed by a three log increase in infectious CT (Figure 17A). A similar trend was observed when cell-associated CT rRNA and CT recovery from penicillin-treated cultures were compared (Figure 17B). There was a marked decrease in the number of infectious CT in the early hours of the infection, while the amount of CT rRNA dramatically increased until it peaked at 25 hours p.i. (Figure 17B). The small peak of CT recovery at 32 hours p.i. temporally followed the peak of CT rRNA, as was observed in untreated cultures (Figure 17A-B). However, unlike

Figure 17

Comparison of the kinetics of recovery of infectious CT to levels of CT rRNA.

A. Control cultures.

B. Penicillin-treated cultures (100 U/ml).



untreated cultures, CT recovery and the levels of CT rRNA from penicillin-treated cultures decreased over time. At 98 hours p.i., the levels of CT rRNA remained positive although CT recovery became undetectable (Figure 17B).

Optimization of PCR

All DNA amplification reactions from CT-infected cell lysates exhibited a band at 144 bp. This band was clearly visible at all dilutions of CT and at all primer concentrations tested. Only a primer band at the gel front was visible from amplification reactions that contained DNA from uninfected McCoy cells. Intensity of bands was not dependent upon primer concentration in the range examined. EcoRI digestion of pooled products confirmed the specificity of the fragment as a diagnostic 103 bp band was generated from CT-positive amplification reactions (Figure 18). This 103 bp band was not detected in amplification reactions from uninfected McCoy cells (Figure 18). For further confirmation of the specificity of this reaction, a southern blot was performed using the internal probe. Hybridization with the 144 bp fragment was observed. Since further experimentation demonstrated that a primer concentration of 0.125 μM was as effective as 0.25 μM, the former concentration was used in all subsequent PCR reactions (Figure 19).

Development of CT Persistently-infected McCoy Cells

Freshly trypsinized McCoy cells were inoculated with CT in suspension and

Figure 18

ECORI DIGESTION OF DNA AMPLIFICATION REACTIONS USING CT-INFECTED McCOY CELL LYSATES AND UNINFECTED McCOY CELL LYSATES. Lane 1 is molecular weight markers. Lane 2 is undigested DNA amplified from CT-infected McCoy cells. Lane 3 is digested DNA amplified from CT-infected McCoy cells. Lane 4 is undigested DNA amplified from uninfected McCoy cells. Lane 5 is digested DNA amplified from uninfected McCoy cells. Arrow indicates 144 bp band. Arrowhead indicates 103 bp band.

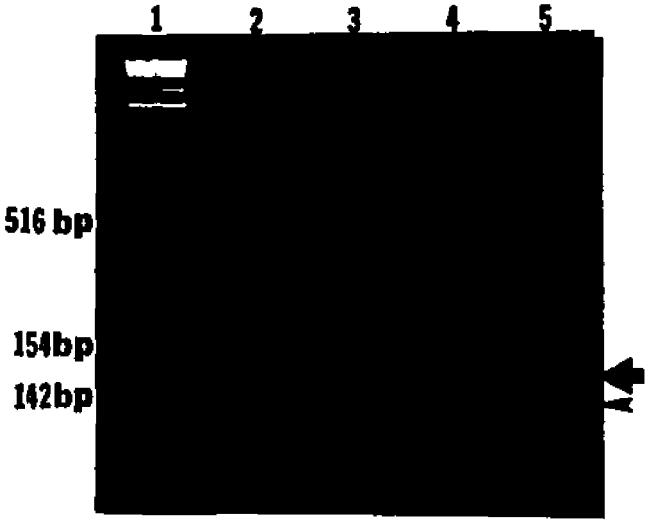
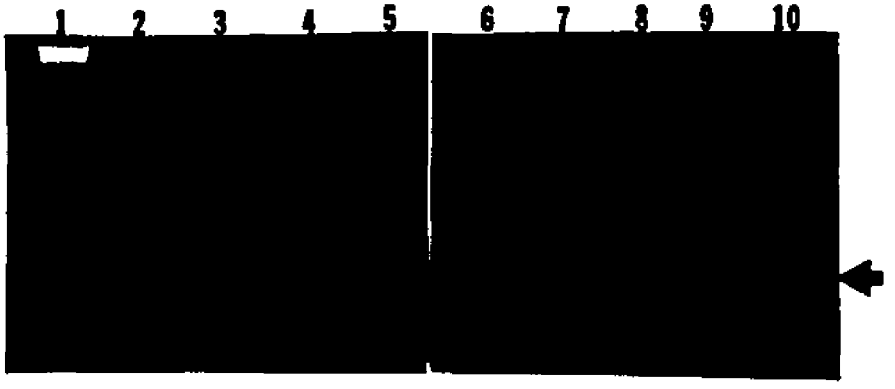


Figure 19

TITRATION OF PRIMER CONCENTRATIONS AGAINST TWO DIFFERENT CONCENTRATIONS OF DNA TO OPTIMIZE PCR CONDITIONS. Lane 1 is molecular weight markers. Lanes 2 through 4 contained 5 μl DNA per reaction. Lane 2 had a primer concentration of 0.25 μM. Lane 3, 0.125 μM. Lane 4, 0.0625 μM. Lane 5, 0.03125 μM. Lanes 6 through 9 contained 2 μl DNA per reaction. Lane 6 had a primer concentration of 0.25 μM. Lane &, 0.125 μM. Lane 8, 0.0625 μM. Lane 9, 0.03125 μM. Lane 9 was the water negative control.

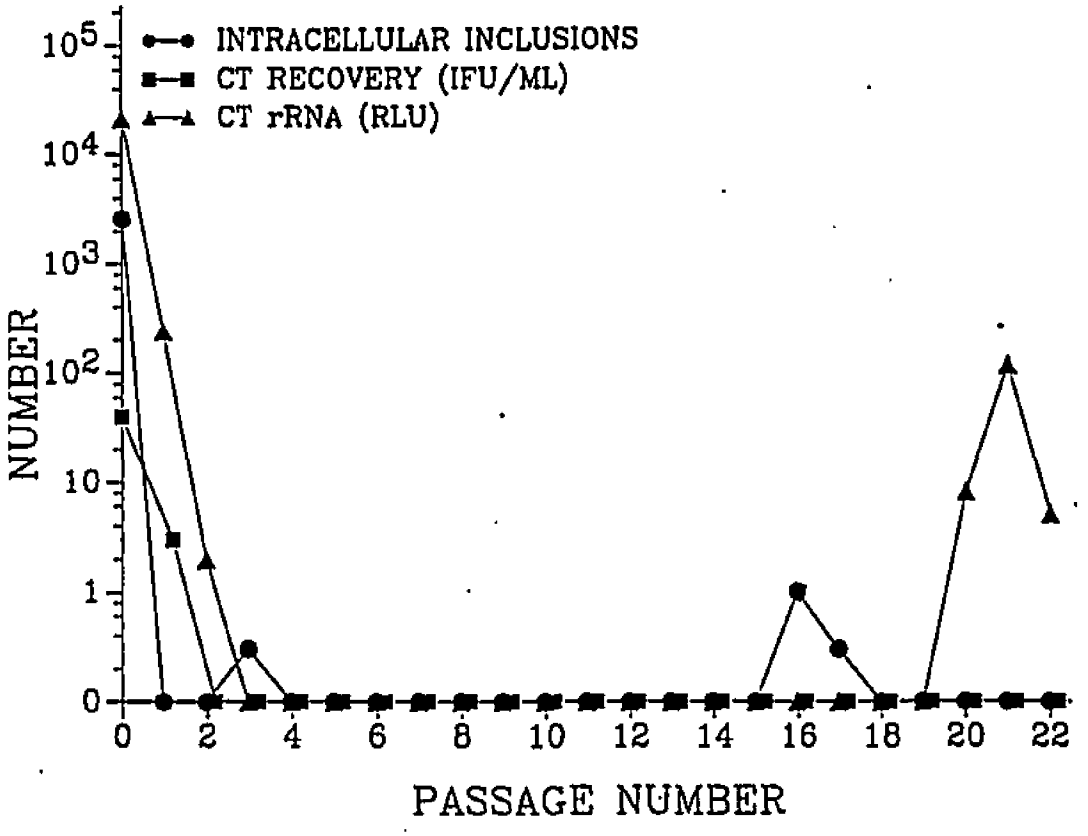


planted in 24-well plates. This primary inoculation was designated as passage 0 (P0). Each subsequent passage was designated sequentially in the same fashion. Three cell lines initiated were: (a) an inoculum of live CT, grown in control medium, (b) an inoculum of live CT, grown in penicillin medium and (c) an inoculum of CT, partially inactivated by UV treatment, grown in control medium. At each subsequent passage, these initial cell lines were placed in either control medium or penicillin medium as indicated. Two lineages of CT-McCoy cells were produced: the penicillin lineage, initially inoculated in penicillin medium and the control lineage, initially inoculated in control medium. The cells inoculated with UV-treated CT were maintained in control medium and formed a separate line (UV-CT-McCoy cells). Only results from the penicillin lineage will be presented in detail.

Penicillin Lineage. The penicillin line of CT-McCoy cells was maintained only in penicillin medium. This cell line was designated PEN. After inoculation during P0, no additional CT was added to this cell line. In the initial passage, a mean of 2581 abnormal intracellular inclusions was detected by iodine stain (Figure 20). These inclusions had the incomplete morphology seen in earlier experiments and pronounced vacuolization of the monolayers was observed. These incomplete inclusions stained with anti-MOMP in the same pattern seen in earlier experiments. During subsequent passages an occasional very small iodine-staining inclusion was observed, but overall, intracellular inclusion formation was not observed while the cells were maintained in penicillin medium (Figure 20). CT recovery from this cell line rapidly decreased from 40 IFU/ml

Figure 20

Kinetics of intracellular inclusion formation, recovery of infectious CT and levels of CT rRNA in the PEN cell line.



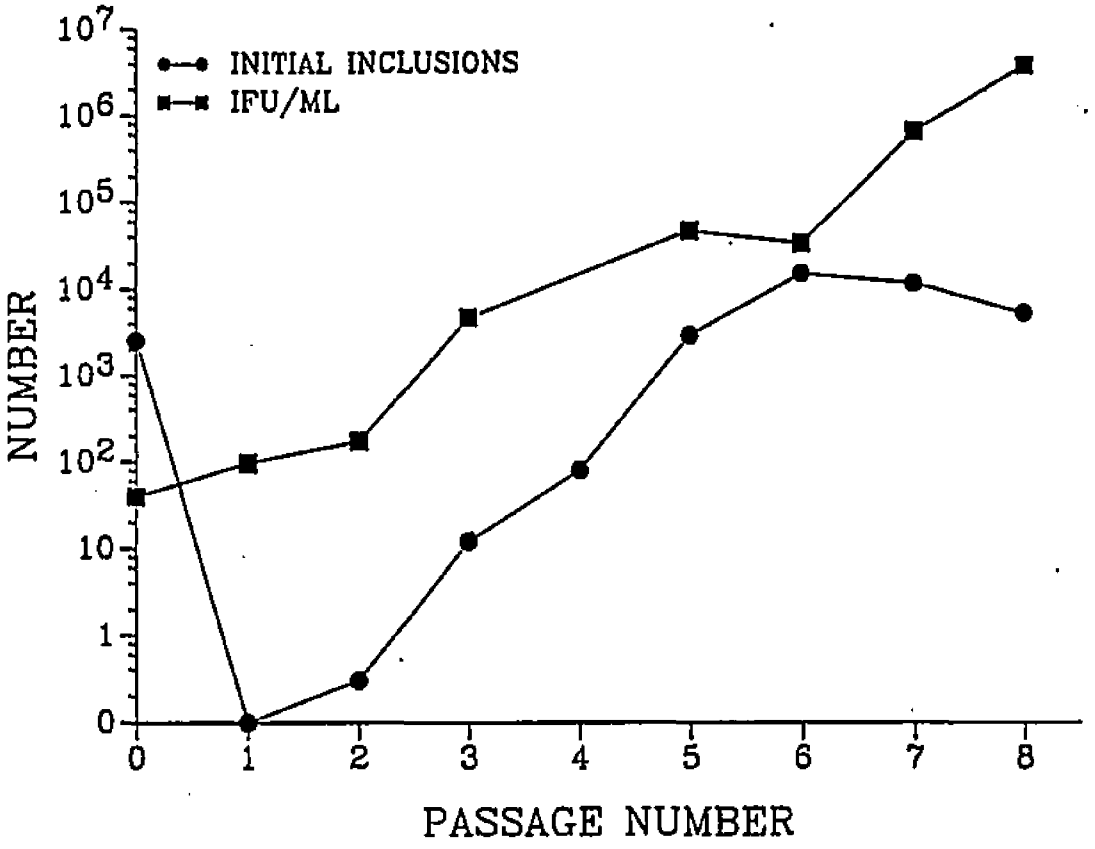
(P0) to 3 IFU/ml (P1) and was not detected in subsequent passages (Figure 20). CT rRNA was detectable in passages 0-2 and at very low levels in passages 20-22, but was undetectable during intermediate passages (Figure 20). The slight increase in rRNA at passages 20-22 was unexpected and the significance of this has not yet been determined.

After the initial passage in penicillin medium, one fraction of cells from the penicillin lineage was cultivated thereafter in control medium. This cell line was designated PEN_1 and was maintained in control medium. Intracellular inclusion formation was zero at P1. However, normal iodine-staining inclusions developed in subsequent passages (Figure 21). In contrast to the PEN line, CT recovery from PEN_1 persisted throughout the experiment (Figure 21). Therefore, presence of penicillin only during the initial passage did not prevent CT replication in subsequent passages.

These results were in contrast to results from other cell lines developed from this lineage. CT-McCoy cells grown in the presence of penicillin for 3, 6, 10, or 18 passages before subsequent cultivation in control medium. These cell lines were designated PEN_3, PEN_6, PEN_{10}, and PEN_{18}, respectively, for the number of passages the cells were maintained in penicillin medium prior to cultivation in control medium for all subsequent passages. The PEN_3 and PEN_6 lines were initially negative by intracellular inclusion formation, recovery of infectious CT and detection of CT rRNA. However, PEN_3 and PEN_6 "recovered" to resume productive CT infection after subsequent cultivation in penicillin-free medium. PEN_3 did not exhibit intracellular inclusion formation for three passages after placement in control medium, but then developed normal iodine staining inclusions. Intracellular inclusions appeared in PEN_3 at passage

Figure 21

Kinetics of intracellular inclusion formation and recovery of infectious CT in the PEN_1 cell line.



6, and infectious CT was produced at passage 5 (Figure 22). CT rRNA was detectable at very low levels during passages 4 and 5, then became negative until passages 10-12, when CT rRNA increased to levels normally detected during active infection (Figure 22). PEN_6 recovered more rapidly than PEN_3. PEN_6 developed intracellular inclusions and produced infectious CT immediately after cultivation in control medium (Figure 23). The levels of CT rRNA in PEN_6 rapidly increased and peaked at more than 500,000 RLU by passage 11 (Figure 23).

CT-McCoy cells grown in penicillin medium for 10 or 18 passages prior to placement in control medium reacted differently than PEN_1, PEN_3 or PEN_6. PEN_{10} exhibited low numbers of very small iodine-staining inclusions from passages 12-20 (Figure 24). Cells on coverslips from passages 13-20 were destained and restained with anti-MOMP. CT MOMP could be detected in these monolayers which indicated that this protein was being expressed. The inclusions tended to be very small and pleomorphic, some had the appearance of complete inclusions and others had the vacuolated ("bubble") appearance of the incomplete inclusions described earlier. No infectious CT was recovered from these cultures even when inclusions were detected in passages 12-20 (Figure 24). Very low levels of CT rRNA were detected after cultivation in control medium, but these levels did not approach the levels seen during active infection (Figure 24). By the parameters examined, PEN_{10} appeared to be a true persistent infection, but it is unknown whether or not further passages would result in spontaneous reactivation and productive infection. PEN_{18} did not exhibit inclusion formation after cultivation in control medium nor was infectious CT recovered (Figure 25). Very low levels of CT

Figure 22

Kinetics of intracellular inclusion formation, recovery of infectious CT and levels of CT rRNA in the PEN$_3$ cell line.

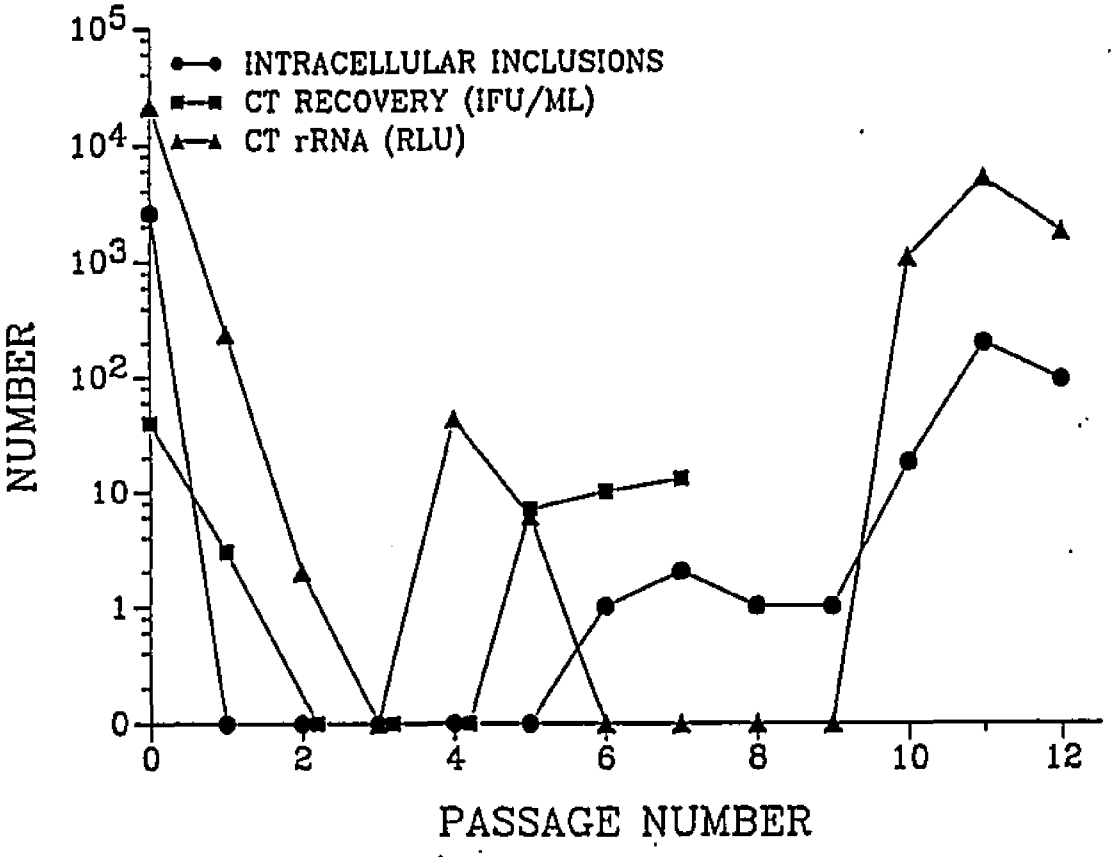


Figure 23

Kinetics of intracellular inclusion formation, recovery of infectious CT and levels of CT rRNA in the PEN_6 cell line.

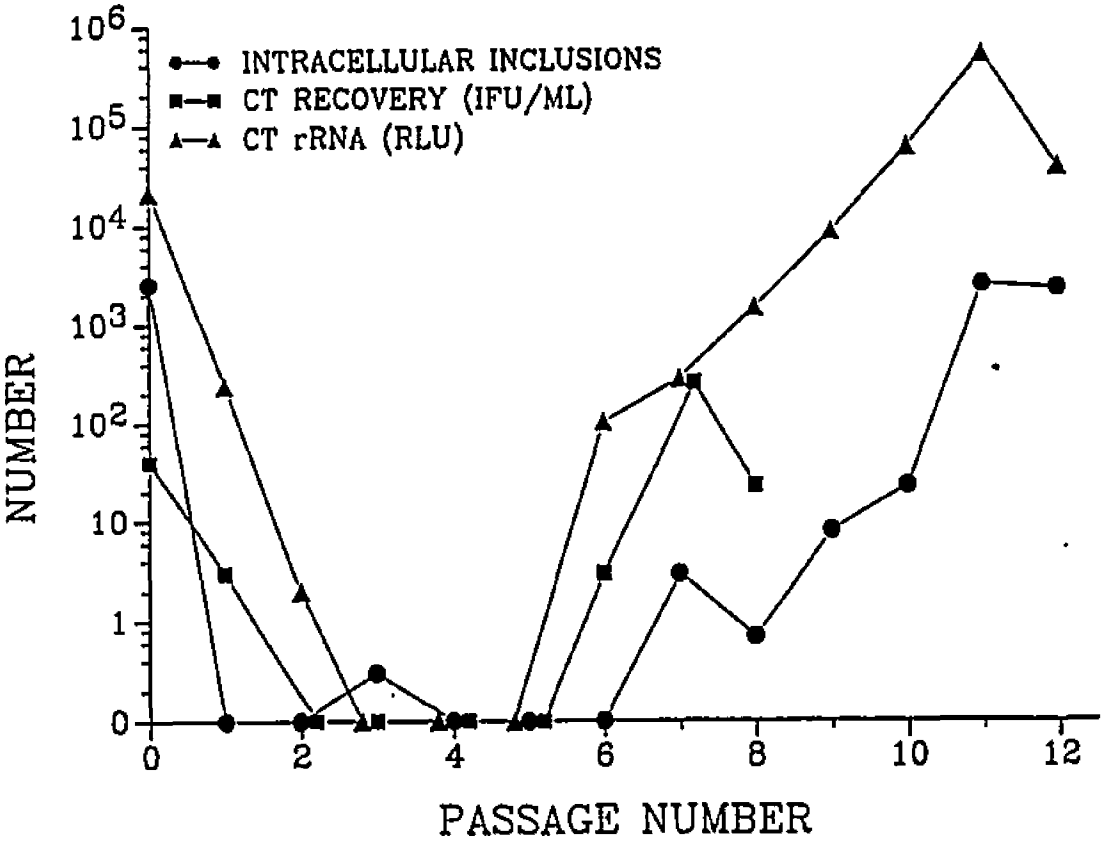


Figure 24

Kinetics of intracellular inclusion formation, recovery of infectious CT and levels of CT rRNA in the PEN_{10} cell line.

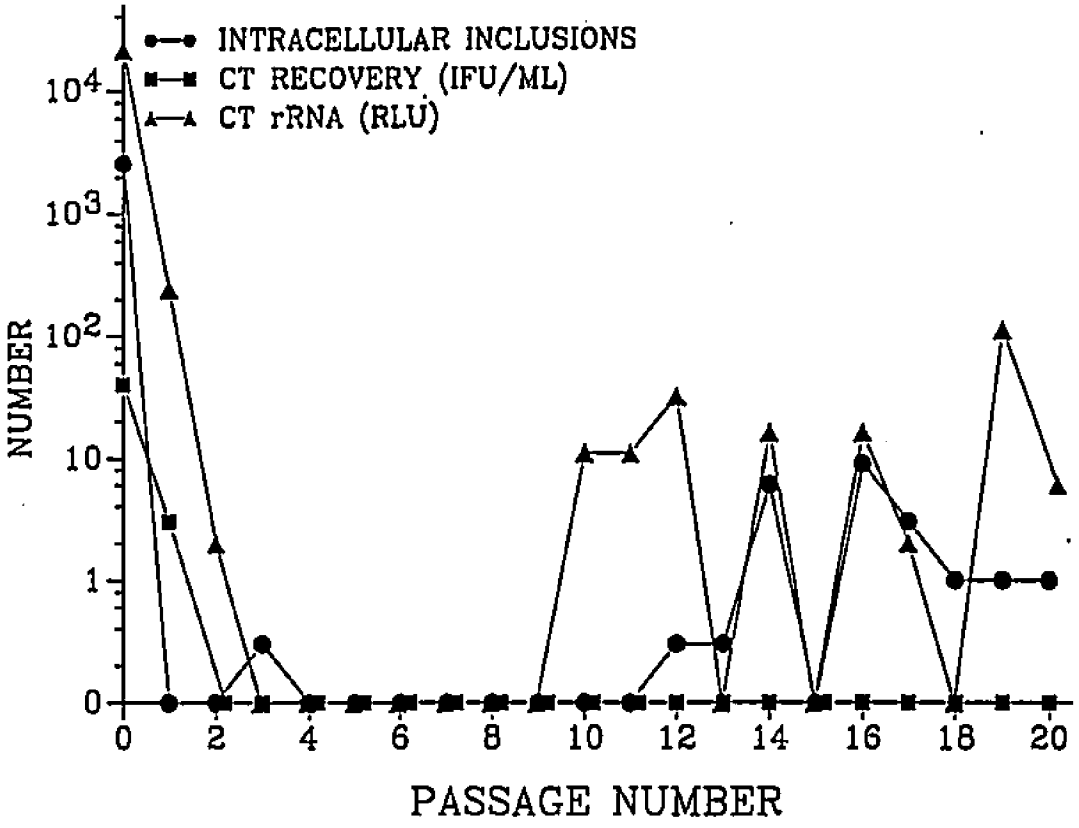
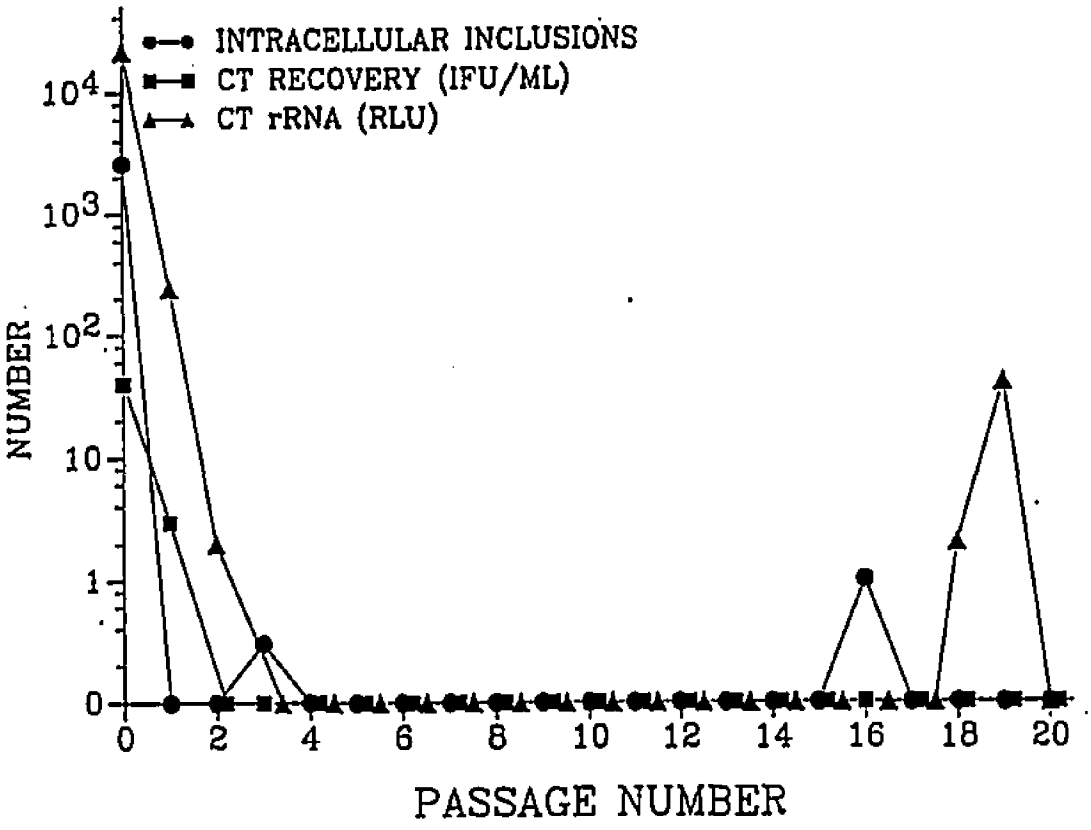


Figure 25

Kinetics of intracellular inclusion formation, recovery of infectious CT and levels of CT rRNA in the PEN_{11} cell line.



rRNA were detected in passages 18 and 19, but none was detected in passage 20 (Figure 25). As with PEN_{10} it is unknown whether or not further culture in control medium would have resulted in spontaneous conversion to a productive infection.

Since continued culture in the presence of a microbiostatic antibiotic may favor selection of resistant strains, it was necessary to determine if such a selection had occurred in this experiment. The PEN_1 cell line was cultivated for 12 passages in the absence of penicillin, after which penicillin was replaced in the culture medium. Intracellular inclusion formation was dramatically inhibited (1 iodine-staining inclusion) in the next two passages. CT recovery decreased from 3.7×10^6 IFU/ml to 0 IFU/ml in passages 13 and 14 (i.e. immediately after penicillin was replaced in the culture medium). Also the level of CT rRNA decreased from 9017 RLU (P12) to 110 RLU after cultivation in penicillin medium. As further evidence that this strain of CT was still sensitive to penicillin, anti-MOMP staining of these monolayers demonstrated the incomplete inclusions seen in earlier experiments. Therefore, the change from persistent to productive infection observed in PEN_1, PEN_3, and PEN_6 was not due to selection of resistant strains of CT, but rather to reactivation of persistent forms.

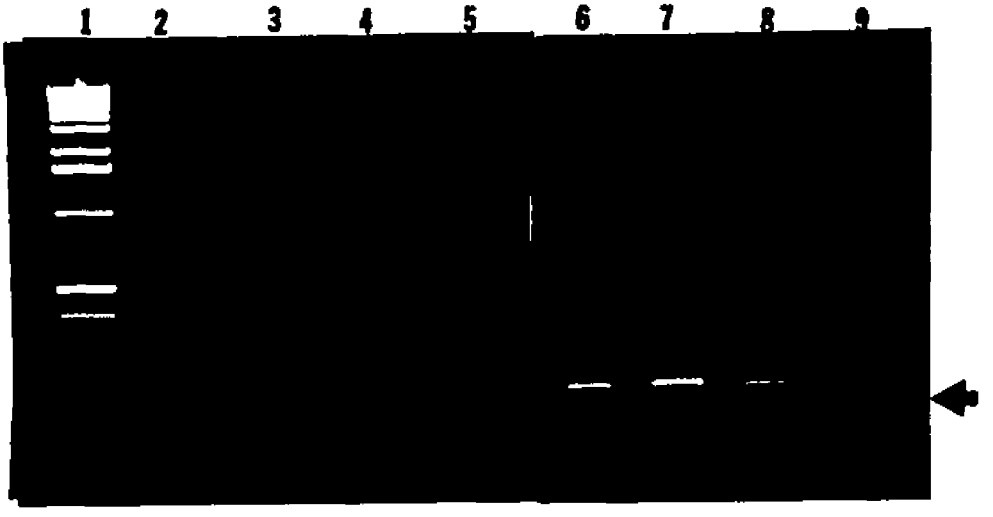
The observation that penicillin-maintained CT-McCoy cell lines can progress from persistent to productive infection suggested that these cells may harbor the chlamydial genome in a latent form since expression was not detected by inclusion formation, recovery of infectious CT, or CT rRNA. PCR seemed an ideal tool to examine this hypothesis. Cell lysates from a single well of each passage of the PEN line of CT-McCoy cells were extracted with phenol/chloroform and ethanol-precipitated to isolate

the DNA. The DNA collected from these extractions underwent PCR using a primer pair that amplifies a 144 bp region in the CT MOMP gene. The chlamydial genome, or at least a portion thereof, was present in all passages of the penicillin line of CT-McCoy cells through passage 15 (Figures 26 and 27). The genome was not detected in passage 16, yet was demonstrated (faint band) in passages 17, 18, and 19 (Figures 27 and 28). The fact that the specific region was not detected in passage 16 but was evident in passages 17-19 may be explained by the fact that DNA from single culture wells was amplified. Cells from the other wells were used for inclusion staining, CT recovery, rRNA determination and continued cultivation. Unless replication allowed progeny or elementary bodies to be distributed to both daughter cells, the cells harboring the CT genome would be diluted slowly, such that one aliquot of cells may contain persistently infected cells while another would not. The fact that dilution appeared to occur suggested that the level of replication in these persistently infected cells was very low and that inclusions may not be transmitted to both daughter cells upon division. The detection of CT DNA in the PEN line further supports the idea that the change from "latent" to productive infection observed in PEN_1, PEN_3, and PEN_6 reflected reactivation of persistent CT infection.

Comparison of the different cell lines of the penicillin lineage of CT-McCoy cell lines revealed two patterns. In the first, three cell lines (PEN_1, PEN_3, and PEN_6) converted to a productive infection developing high numbers of intracellular inclusions (Figure 29). A pattern of cycling was observed with some cell lines (Figure 29). For example, at passage 15, the PEN_6 monolayer began to deteriorate which resulted in the

Figure 26

AMPLIFICATION REACTIONS USING DNA FROM THE PEN CELL LINE. Lanes 1 and 10 are molecular weight markers. Lanes 2 through 9 are amplification reactions using DNA from sequential passages of the PEN cell line. Lane 2, passage 1. Lane 3, passage 2. Lane 4, passage 3. Lane 5, passage 4. Lane 6, passage 5. Lane 7, passage 6. Lane 8, passage 7. Lane 9, passage 8. Lane 11 is the positive control. Lane 13 is the negative control. Lane 14 is the water negative control. Arrow indicates position of 144 bp band.



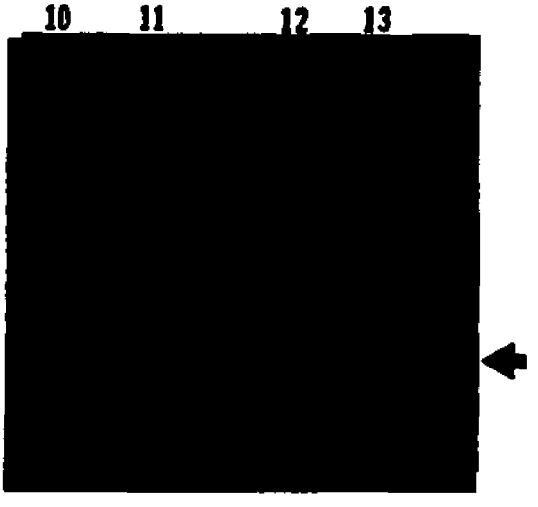


Figure 27

AMPLIFICATION REACTIONS USING DNA FROM THE PEN CELL LINE. Lanes 1, 6, 11 and 16 are molecular weight markers. Lane 2 is the water negative control. Lane 3 is the negative control. Lane 4 is the positive control. Lanes 5 through 13 are amplification reactions using DNA from sequential passages of the PEN cell line. Lane 5, passage 9. Lane 7, passage 10 Lane 8, passage 11. Lane 9, passage 12. Lane 10, passage 13. Lane 12, passage 14. Lane 13, passage 15. Lane 14, passage 16. Lane 15, passage 17. Lanes 16 through 23 contain products of an ECORI digestion of positive and negative controls. Lane 16 is a molecular weight marker. Lane 17 is undigested water negative control. Lane 18 is digested water negative control. Lane 19 is undigested negative control. Lane 20 is digested negative control. Lane 21 is undigested positive control. Lane 22 is digested positive control. Lane 23 is digested pGEM-IL1 as a positive control for activity of the enzyme. Arrows indicates position of 144 bp band. Arrowhead indicates position of the 103 bp band.

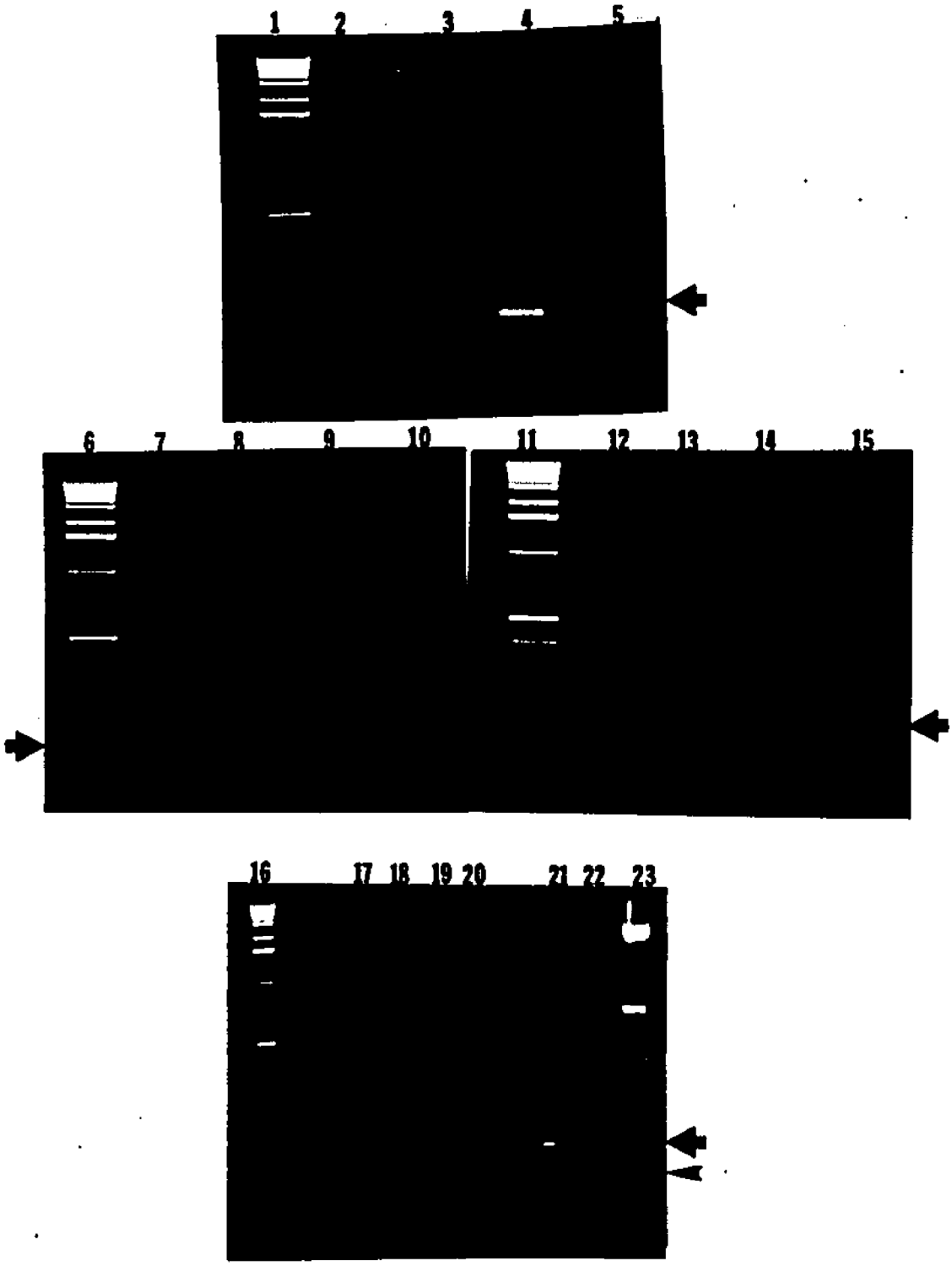

1 2 3 4 5
6 7 8 9 10
11 12 13 14 15
16 17 18 19 20 21 22 23

Figure 28

AMPLIFICATION REACTIONS USING DNA FROM THE PEN CELL LINE. Lane 1 is molecular weight markers. Lane 2 is the positive control. Lane 3 is the water negative control. Lane 4 is the negative control. Lanes 5 through 7 are amplification reactions using DNA from sequential passages of the PEN cell line. Lane 5, passage 18. Lane 6, passage 19. Lane 7, passage 20. Arrow indicates position of 144 bp band.

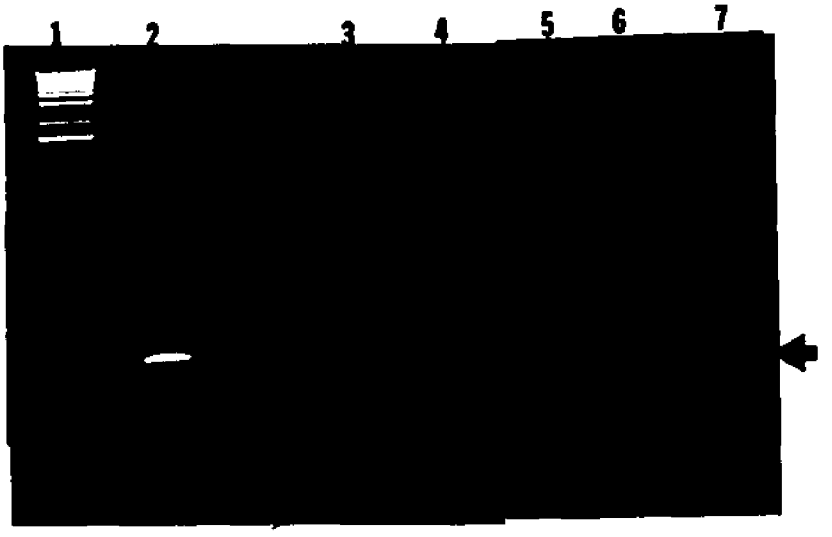
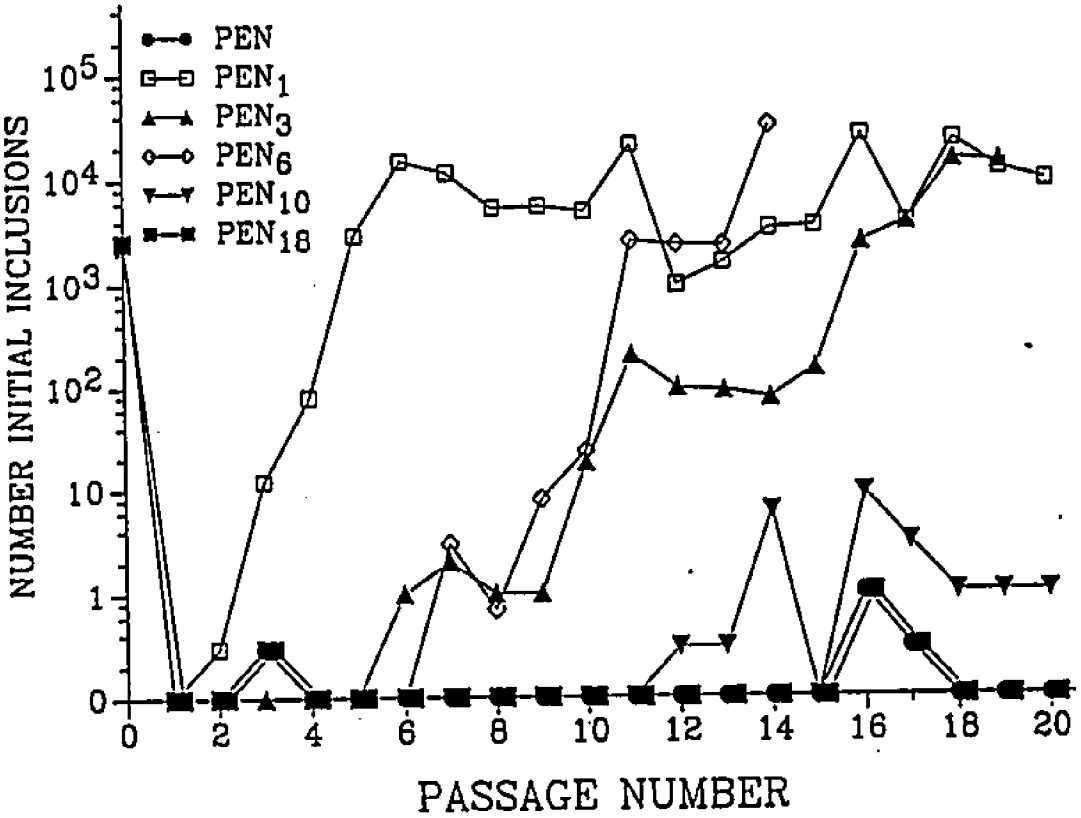


Figure 29

Comparison of the kinetics of intracellular inclusion formation in the penicillin lineage.



"apparent" disappearance of intracellular inclusions. Further cultivation resulted in regeneration of the monolayers and then another cycle of destruction occurred. In the second pattern, PEN_{10} began to produce low levels of intracellular inclusions (Figure 29) but these levels remained low and the inclusions tended to be very small. Neither PEN_{10} nor PEN_{18} produced infectious CT (Figure 30) during the time studied, although both lines exhibited very low levels of CT rRNA (Figures 31). The very low levels of CT rRNA detected in PEN_{10} and PEN_{18} were at the threshold of detection and do not compare to the rapidly increasing levels of CT rRNA found in PEN_3 and PEN_6 (Figure 31). Further work is required to determine if these cell lines could be reactivated by continued culture or by treatment with hormones or cytokines. These cell lines may prove to be the best model to study the mechanism(s) underlying CT persistence and reactivation.

Control Lineage. CT persistently-infected McCoy cell lines were produced without the use of penicillin. McCoy cells inoculated with a high MOI remained healthy and viable for several passages but would then undergo a cycle of destruction with lysis of most of the monolayer. Further cultivation in fresh medium allowed the monolayers to become healthy again, only to undergo another cycle of CPE in subsequent passages. By passage 11, a stable productive infection was established and was maintained for an extended period of time. UV-treatment of CT allowed for inoculation of McCoy cells with a very low MOI (MOI = 9×10^{-4}). Inoculation of the cells with a low MOI allowed a stable productive infection to be established without destruction of the

Figure 30
Comparison of the kinetics of infectious CT production in the penicillin lineage.

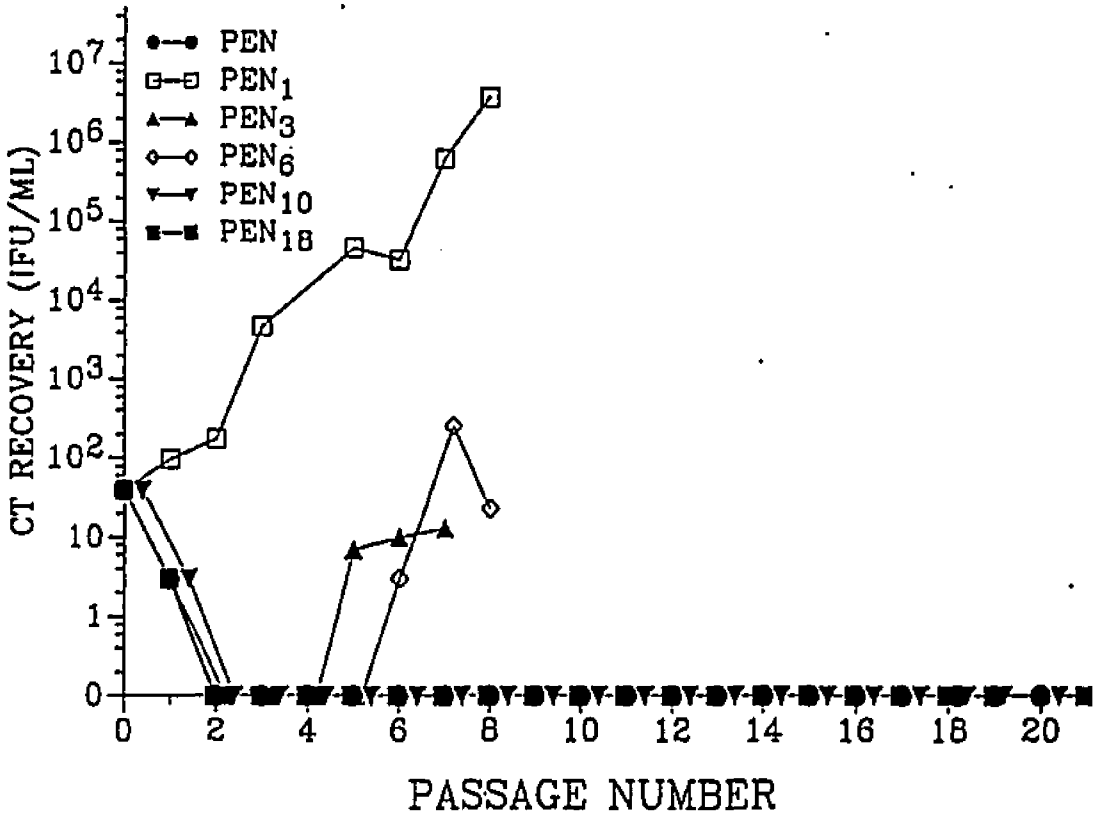
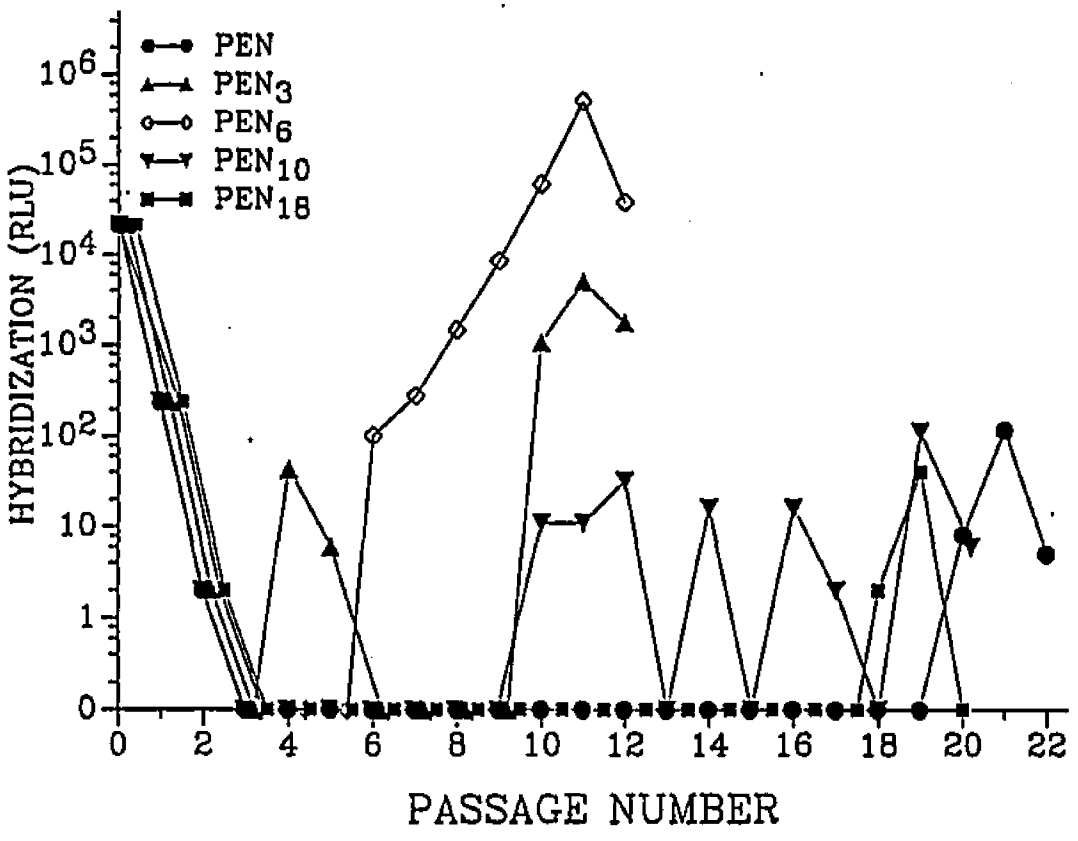


Figure 31

Comparison of the kinetics of the levels of CT rRNA from the different cell lines of the penicillin lineage.



monolayer. It was interesting to note that the UV-CT-McCoy cells were negative by all parameters examined for the first few passages, but began to develop intracellular inclusions and infectious CT in subsequent passages. Control CT-McCoy cells and UV-CT-McCoy cells warrant further examination as examples of persistent CT infection established without the use of an antibiotic.

In Vivo Models

Experiment 1

Virgin female C3H mice were pretreated with DP or remained untreated prior to inoculation with CT or mock-inoculation with uninfected McCoy cell lysate. After inoculation, vaginal swabs from CT-infected mice were collected sequentially and cultured for infectious CT. CT-inoculated mice which had not been pretreated with DP transiently shed CT vaginally, although 50% of these inoculated mice harbored CT in the upper tract (Table 2). A more detailed depiction of CT recovery from these mice is shown in Table 3. One mouse, sacrificed on day 43 p.i., had not shed CT vaginally for 33 days, yet infectious CT was isolated from the inoculated uterine horn. Table 3 shows that one animal sacrificed on day 15 p.i. had ceased to shed CT vaginally yet infectious CT was isolated from six tissues of the upper tract (inoculated and uninoculated sides). Therefore, a negative vaginal swab did not correlate with elimination of CT from the upper tract. Gross pathology noted in the nontreated CT-inoculated mice included

Table 2
CT Culture of Vaginal Swabs Collected Sequentially from CT-Inoculated Mice
(No DP-Pretreatment)

MOUSE NO.	DAYS POST-INOCULATION[A] 2	7	10	13	15	20	24	29	34	38	41	43	CT FROM UPPER TRACT	GROSS PATHOLOGY[B]
1-1	—	—	—	—	—								NO	ND
1-2	—	+	+	—	—								YES	ND
1-3	—	—	—	+	—								YES	ND
1-4	—	—	+	—	—	—	—	—	—	—	—	—	NO	YES(H)
1-5	—	—	—	+	—	+	—	—	—	—	—	—	NO	YES(BH;++)
1-6	—	—	+	—	—	—	—	—	—	—	—	—	YES	YES(H;++)

[A] + = CT isolated from vaginal swab.
— = CT was NOT isolated from vaginal swab.
CT was not isolated from any of the mock-inoculated animals at either 2 days p.i. or at time of sacrifice.

[B] H = Hydrosalpinx. BH = Bilateral Hydrosalpinges.
+-++++ = Increasing severity of erythema. ND = Not Detected.

Table 3 CT Isolation From Upper Tract Tissues of CT-Inoculated Mice
(No DP-Pretreatment)

Animal No.	Days P.I.	Inoculated Side			Uninoculated Side			UB
		Ovary	Tube	Horn	Ovary	Tube	Horn	
1-1	15	—	—	—	—	—	—	—
1-2	15	—	—	—	—	+	+	—
1-3	15	—	+	+	+	+	+	+
1-4	43	—	—	—	—	—	—	—
1-5	43	—	—	—	—	—	—	—
1-6	43	—	—	+	—	—	—	—

+ = CT was isolated from the tissue.
— = CT was NOT isolated from the tissue.
UB= Uterine Body.

hydrosalpinx formation and erythema (Table 2). Isolation of CT from the upper tract did not correlate with detectable presence or absence of gross pathology. Some mice that were positive for CT did not have detectable gross pathology, while other mice, which exhibited gross pathologic damage, did not seem to harbor CT (Table 2). Histopathologic examination of the tissues from these mice has not yet been completed, but should reveal whether or not damage at the microscopic level had occurred in untreated CT-inoculated mice.

In contrast, DP-pretreated CT-inoculated mice exhibited more extensive vaginal CT colonization, as CT was recovered with greater frequency from successive vaginal cultures (Table 4). A number of these mice underwent recurrent vaginal shedding of CT ($\geq$2 successive negative vaginal cultures followed by a positive culture). In fact 58% of the DP-pretreated CT-inoculated mice sacrificed at later times (43 and 84 days p.i.) underwent recurrent vaginal shedding of CT. Post-surgical adhesions were noted frequently in both mock-inoculated and CT-inoculated mice, yet hydrosalpinx formation, erythema and enlarged fallopian tubes were found only in CT-inoculated mice and were scored as CT-specific gross pathology. In the DP-pretreated CT-inoculated mice, 73.3% of the mice exhibited detectable gross pathology at the time of sacrifice (Table 4). Of the 3 mice sacrificed at day 84 p.i., one had a hydrosalpinx in the absence of detectable CT in vaginal secretions and upper tract (Table 4). Histologic examination of the tissues from these mice has not yet been completed, but should reveal the presence and type of damage that might have occurred at the microscopic level.

Early in the infection (15 days p.i.) only 2/12 DP-pretreated mice exhibited

Table 4 CT Culture of Vaginal Swabs Collected Sequentially From CT-Inoculated Mice (DP-Pretreated)

MOUSE NO.	DAYS POST-INOCULATION[A] 2	7	10	13	15	20	24	29	34	38	41	43	45	49	52	55-84	CT FROM UPPER TRACT	GROSS PATHOLOGY[B]
3-3	+	+															YES	ND
3-4	+	+															YES	ND
3-9	+	+															YES	YES(H;+++)
2-2	+	+	—	+	—												YES	YES(H;+)
2-3	+	—	—	—	—												YES	YES(E)
2-4	+	+	+	+	+												YES	YES(E;+)
2-1	+	+	—	+	+	—	+	+									YES	YES(H;++)
2-5	+	+	—	—	—	+	+	+									YES	YES(++)
2-6	+	+	+	—	—	+	+	+									YES	YES(BH;+++)
3-2	+	+	+	+	—	—	—	+	—	+	+	+					YES	YES(+)
3-5	+	+	+	—	—	—	—	—	—	+	+	+					YES	YES(BH;+)
3-6	+	+	+	+	—	—	—	+	—	+	+	—					YES	YES(H)
3-1	+	+	+	+	—	+	+	+	—	—	—	—	—	—	—	—	NO	ND
3-7	+	+	—	+	—	+	—	—	—	—	—	+	—	—	—	—	NO	ND
3-8	+	+	—	+	—	—	—	—	—	+	+	+	+	+	+	—	NO	YES(H)

[A] + = CT isolated from vaginal swab. — = CT was NOT isolated from vaginal swab.
CT was not isolated from any of the mock-inoculated animals at either 2 days p.i. or at time of sacrifice.

[B] H = Hydrosalpinx. BH = Bilateral Hydrosalpinges. E = Enlarged Fallopian Tubes. ND = Not Detected.
+-+++ = Increasing severity of erythema.

vaginal shedding of CT. Of the three mice sacrificed at this time, none yielded a positive vaginal culture, yet all three harbored CT in multiple tissues of the upper tract (Tables 4 and 5). This supported previous findings that a negative vaginal culture did not correlate with elimination of CT from the upper tract. CT was not isolated from vaginal secretions or from upper tract tissues of three mice sacrificed at 84 days p.i. (Table 4). It has not yet been determined if these mice harbored CT in a form of persistent infection undetectable by culture. In fact, if the different patterns of vaginal shedding of CT were compared to isolation of CT from the upper tract, four main motifs were possible: a.) CT isolation from upper tract tissue(s) and vaginal secretions, b.) CT isolation from the vagina but not from tissue(s) of the upper tract, c.) CT isolation from the tissue(s) of the upper tract but not from the vagina and d.) CT isolation from neither vaginal secretions nor from tissue(s) of the upper tract. Three of these motifs were observed (Table 6A). No CT-inoculated mice had a positive vaginal culture without isolation of CT from tissue(s) of the upper tract. If all time points in the experimental design were considered, 28.6% of the mice had a "persistent" infection, i.e. negative by vaginal culture yet harboring CT in the upper tract (Table 6A). If only the time points 2, 4, and 6 weeks p.i. were considered 33.3% of the mice harbored a "persistent" infection (Table 6B). DP-pretreatment enhanced vaginal shedding of CT. However, 50% of nontreated mice had a "persistent" infection (Tables 6A and 6B). This was in contrast to DP-pretreated CT-inoculated mice of which only 20% had a "persistent" infection (Table 6A). According to these results, a valuable model to study persistent infection would be CT inoculation of mice that had not been pretreated with DP, with

Table 5 CT Isolation From Upper Tract Tissues of CT-Inoculated Mice (DP-Pretreated)

Animal No.	Days P.I.	Inoculated Side			Uninoculated Side			UB
		Ovary	Tube	Horn	Ovary	Tube	Horn	
3-3	7	+	+	+	—	—	—	+
3-4	7	+	+	+	—	+	+	+
3-9	7	—	—	+	—	+	—	+
2-2	15	—	+	+	—	—	+	—
2-3	15	—	+	+	—	+	+	+
2-4	15	—	+	+	—	+	+	—
2-1	29	—	—	+	—	—	+	+
2-5	29	—	—	—	—	+	+	+
2-6	29	+	—	+	+	—	+	+
3-2	43	—	—	—	—	+	+	—
3-5	43	—	—	—	—	—	+	—
3-6	43	—	+	—	—	—	—	—
3-1	84	—	—	—	—	—	—	—
3-7	84	—	—	—	—	—	—	—
3-8	84	—	—	—	—	—	—	—

+ = CT was isolated from the tissue.
— = CT was NOT isolated from the tissue.
UB= Uterine Body.

Table 6 Evidence for Persistent Chlamydial Infection of C_3H Mice

A

CT Recovery[A]		Number of Mice		
Vaginal	Upper Tract	Progesterone Pretreatment	No Pretreatment	Total
YES	YES	9	0	9
YES	NO	0	0	0
NO	YES	3	3	6
NO	NO	3	3	6

[A] At time time of sacrifice (1, 2, 3, 6 and 12 weeks post-inoculation)

B

CT Recovery[B]		Number of Mice		
Vaginal	Upper Tract	Progesterone Pretreatment	No Pretreatment	Total
YES	YES	6	0	6
YES	NO	0	0	0
NO	YES	3	3	6
NO	NO	0	3	3

[B] 2, 4, 6 weeks post-inoculation

observation and sampling of such mice for 2-6 weeks p.i.

CT was never isolated from a mock-inoculated mouse. No other bacterial pathogens were detected from the routine bacterial cultures of the peritoneal swabs collected at the time of sacrifice from CT- or mock-inoculated mice which suggested that no other bacterial infection had occurred, and served as a control for the inoculation procedure.

Experiment 2

Since penicillin treatment of CT-infected McCoy cells resulted in establishment of persistent CT infection in vitro, the effect of penicillin upon CT infection in vivo was examined. DP-pretreated C3H mice were inoculated with CT as previously described. At days 9 and 16 p.i., 2000 U of penicillin G were injected i.m into 34 of the CT-inoculated mice; 32 CT-inoculated mice remained unmedicated. The dosage of penicillin G used was the equivalent of a 2,000,000 U injection of penicillin G in a human. One of the unmedicated CT-inoculated mice died during the experiment and was excluded from subsequent calculations. As in experiment 1, sequential vaginal swabs were collected and cultured for CT. Injection of penicillin did not halt vaginal shedding of CT since 61.3% of CT-inoculated mice were culture-positive for CT on day 10 p.i., and 35.3% were positive for CT on day 17 p.i. Of the unmedicated CT-inoculated mice, 50% were positive for CT on day 10 p.i., while none were positive for CT on day 17 p.i. Injection of penicillin did alter the pattern of vaginal CT shedding as only 64.7% of the penicillin-treated mice had recurrent vaginal shedding as compared to 90.3% of

the unmedicated, CT-inoculated mice (Table 7).

At 2 weeks p.i., only 16.7% (Experiment 1) and 19.4% (Experiment 2) of the unmedicated CT-inoculated mice were positive for CT as compared to 62.5% of the penicillin-treated CT-inoculated mice (Table 8). At 2 weeks p.i., some critical event seemed to have occurred that resulted in the apparent "elimination" of CT from the vaginal vault of unmedicated CT-inoculated mice. This elimination of CT from the vaginal vault was also observed in nontreated, CT-inoculated mice as 0/6 were CT culture-negative at 2 weeks p.i. Penicillin treatment somehow modified this response, since vaginal CT shedding was not suppressed at 2 weeks p.i. in penicillin-treated CT-inoculated mice.

At 10 weeks p.i., mice that had been CT-negative for $\geq$2 successive cultures were manipulated in an attempt to reactivate vaginal CT shedding (first reactivation group). This subpopulation of mice was injected with CA or DP, while other mice from this subgroup injected with saline served as controls. Of the penicillin-treated, CT-inoculated mice injected with CA, 1/7 (14%) exhibited transient reactivation of vaginal CT shedding (Table 9). Of the unmedicated, CT-inoculated mice that underwent the induction regimen, 28% injected with CA and 33% injected with DP had a transient reactivation of vaginal CT shedding (Table 9). Reactivation was not observed in any of the mice injected with saline (Table 9). The reactivation events were transient and only very low numbers of inclusions were observed (<20/coverslip). At 16 weeks p.i. (5 weeks after induction), the first reactivation group was sacrificed and the upper tract tissues were cultured for CT. Infectious CT was not isolated from any animal.

Table 7 Recurrent Vaginal Shedding of CT Indicates Persistent CT Infection in C3H Mice

Therapy	Recurrent Vaginal Shedding		Gross Pathology	
	No. Recurrent[A] / No. Inoculated	(%)	No. Positive / No. Inoculated	(%)
Penicillin[B]	22/34	(64.7)	24/34	(70.6)
None	28/31	(90.3)	22/31	(71.0)

[A] Recurrent vaginal shedding was defined as positive CT culture obtained after 2 or more successive negative cultures; although all mice inoculated eventually became negative.

[B] 2,000 U i.m., days 9 and 16

Table 8 Comparison of In Vivo Experiments

Experiment Number	Pretreatment	Therapy	No. Positive/No. Inoculated						
			Total No. Positive[A] / No. Inoculated	Weeks Post-Inoculation					
				1	2	4	6	12	22
1	None	None	5/6	1/6	0/6	0/3	0/3	ND[B]	ND
	Depo-Provera	None	15/15	14/15	2/12	6/9	2/3	0/3	ND
2	Depo-Provera	None	31/31	27/28[C]	6/31	20/30[D]	21/31	0/12	0/12
	Depo-Provera	Penicillin[E]	34/34	32/34	20/32[F]	18/34	19/34	1/13	0/13

[A] Number of mice exhibiting vaginal CT shedding $\geq$1 time.
[B] Not done
[C] Contaminant could not bee eliminated from cultures of three mice.
[D] Contaminant could not be eliminated from the culture of one mouse.
[E] 2,000 U penicillin i.m., days 9, and 16
[F] Contaminant could not be removed from the cultures of two mice.

Table 9 Reactivation of CT Demonstrates Persistent CT Infection

Therapy	Reactivation of Vaginal CT Shedding[A]			Gross Pathology	
	Agent	No. CT Positive / No. Induced	(%)	No. Gross / No. Inoculated	(%)
Penicillin[B]	Cortisone-Acetate	1/7	(14)	6/7	(85.7)
	Depo-Provera	0/7	(0)	5/7	(71.4)
	Saline	0/7	(0)	3/7	(42.8)
None	Cortisone-Acetate	2/7	(28)	3/7	(42.8)
	Depo-Provera	2/6	(33)	5/6	(83.3)
	Saline	0/6	(0)	5/6	(83.3)

[A] Animals were induced at 10 weeks p.i. Only mice which had been negative for ≥ 2 successive cultures were induced.
Cortisone-Acetate (125 mg/kg, s.c., every other day for 12 days)
Depo-Provera (2.5 mg, s.c., 2 injections seven days apart)
Saline (0.2 ml, s.c., every other day for 12 days)

[B] 2,000 U i.m., days 9 and 16

Of the remaining mice not included in the first reactivation group, those that had become CT-negative for ≥ 2 successive cultures were subjected to the induction regimen at 17 weeks p.i. None of the mice, from either the penicillin-treated group or the unmedicated group, became positive for CT. These mice were sacrificed at 22 weeks p.i. (5 weeks after induction). Infectious CT was not isolated from any of these animals. Further examination of the tissues from these mice, using molecular methods such as in situ hybridization or PCR, must be performed to determine if CT was eliminated from these animals or if it remains sequestered in a persistent or latent form. At the time of sacrifice, gross pathologic evidence of damage was observed in 70.6% of the penicillin-treated mice and in 71.0% of the unmedicated mice (Table 7). Although infectious CT was not isolated from the tissues of these mice, tissue damage had occurred. Penicillin treatment did not protect the CT-inoculated mice from this damage (Table 7). Further histopathologic examination of tissues from these mice needs to be performed to determine the extent and type of damage, and to determine if penicillin-treatment altered either of these parameters.

No pathogenic bacteria were isolated from either the aerobic or anaerobic cultures of peritoneal swabs collected at the time of sacrifice.

CHAPTER 4

Discussion

The mechanism(s) of persistent CT infection are poorly understood. Using in vitro and in vivo models, persistent CT infection was examined at the molecular and cellular level. The unusual effect of penicillin upon CT infection in vitro has been noted in previous literature (Kramer and Gordon 1971, Johnson and Hobson 1977, Hobson et al. 1982). Abnormal, abortive inclusion formation was noted by Johnson and Hobson (1977) when penicillin-treated CT-infected cells were stained with giemsa and examined using dark field microscopy. These "abortive" inclusions resembled the morphology of the abnormal intracellular inclusions observed in penicillin-treated CT-infected McCoy cells stained with iodine observed in the current study (Figure 3B). As only large vacuoles were observed when these cells were stained with iodine, indicating that little or no glycogen deposition had occurred in the abnormal inclusions. The significance of this is unknown although it has been suggested that glycogen deposition in CT inclusions is not required for complete replication (Matsumoto 1988). The presence of CT MOMP and CT LPS in the abnormal inclusions suggests that penicillin inhibited neither CT EB entry nor reorganization to RB form. This supported earlier ultrastructural analysis that had revealed RB formation in penicillin-treated CT-infected cells, however in that study the RB forms were abnormally large (Kramer and Gordon 1971). The unusual deposition of CT MOMP and CT LPS (Figure 4B) suggests that the RB formation in the penicillin-treated CT-infected cells was different from that in untreated cultures, however

ultrastructural analysis was not performed. The appearance of CT MOMP and CT LPS in the abnormal inclusions also suggests that penicillin treatment did not inhibit the synthesis of either of these antigens. Further biochemical analysis needs to performed to determine if these components have been altered.

Penicillin pretreatment of McCoy cells did not render the cells unable to support complete CT replication as even cells grown in penicillin for two weeks allowed full CT replication provided that penicillin was removed prior to inoculation (Figures 5 and 6). This result supports earlier work which showed that penicillin preincubation of McCoy cells did not result in loss of the ability of this cell line to support CT replication (Johnson and Hobson 1977). In the current study, intracellular levels of penicillin did not inhibit CT inclusion formation. This is in contrast to one earlier report that stated that in HeLa cells 4 pmol/ml penicillin could inhibit CT replication (Barbour et al. 1982). In the same study, an intracellular concentration of 52 nmol/ml penicillin that was reported (Barbour et al. 1982). The difference between these results may be due to differences in penicillin uptake between McCoy cells and HeLa cells, however, uptake studies were not performed. All experiments in the present study, where penicillin was removed and the monolayers washed prior to inoculation, no inhibition of subsequent CT replication was seen.

In the range of penicillin concentrations examined (10 U to 200 U/ml), there was no dose dependent response of intracellular inclusion formation or recovery of infectious CT (Figures 8 and 11). Although the range chosen included values above and below a published MIC value, it was apparently too concentrated to allow detection of a dose

dependent response (Lee et al. 1978). Reported MIC values for penicillin against CT vary widely depending upon the technique chosen (Blackman et al. 1977, Lee et al. 1978, Hobson et al. 1982 , How et al. 1985). Several studies have demonstrated a dose dependent response in the penicillin concentration range of 0.01 to 10 U/ml (Blackman et al. 1977, Johnson and Hobson 1977). In the current study, initial inclusion formation was not inhibited although the morphology of the inclusions was altered. Furthermore, addition of penicillin to CT cultures drastically reduced yield of infectious CT. The MOI was a factor in these experiments. At a high MOI (e.g. 2.0) penicillin did not completely inhibit CT replication although recovery was reduced greater than 1,000,000-fold (Figure 10). Penicillin treatment inhibited RB maturation into EB, resulting in the production of defective EBs, or in the creation of some other noninfectious form of CT.

Although little or no infectious CT was produced in the penicillin-treated cultures, it is possible that noninfectious RBs or defective EBs were present. Since culture would not detect either of the latter, a cDNA probe specific for CT rRNA was employed. The limit of detection of the probe is 5 IFU. Additionally, the probe detects UV-inactivated and live CT with the same sensitivity (Figure 14). Using this probe, the kinetics of CT rRNA in untreated and penicillin-treated CT cultures were compared to levels of infectious CT detected by culture. This study examined the kinetics of acute CT infection in CH-treated McCoy cells. Recovery of infectious CT from supernatants or cell lysates from untreated cultures exhibited a typical growth curve with evidence of complete CT replication (Figure 15A). However, the growth curve obtained from cell lysates of penicillin-treated CT cultures was very different from that of the control, with

a small peak of IFU production at 32 hours p.i., gradually decreasing until no infectious CT were detected at 98 hours p.i.(Figure 15B). This small peak of IFU production could represent either an abortive attempt at normal replication or residual inoculum. Since no IFU were detected in glass controls at 32 hours p.i., it could be argued that this small peak of IFU production was not due to residual inoculum, but this is yet to be substantiated.

The kinetics of CT rRNA levels in untreated and penicillin-treated cultures was examined. CT rRNA was detected in supernatants of untreated and penicillin-treated CT cultures, although lower concentrations were observed in the penicillin-treated CT-infected cultures (Figure 16A). The CT rRNA in supernatants reflected either release of CT EBs into the culture medium or release of RBs due to cell lysis. As culture was not performed on supernatants of penicillin-treated cultures, it was not possible to determine if the cell-free CT was infectious. Although this result demonstrated that some form of CT was released into supernatants of penicillin-treated CT cultures, the existence of defective EBs was neither proved nor disproved.

Cell-associated CT rRNA was detected in untreated and penicillin-treated CT cultures, however with two different profiles. Cell-associated recovery from control cultures showed the expected decrease in early hours of infection when infectious EBs mature into noninfectious RBs (Figure 15B). The maturation into RBs was supported by the dramatic increase in the amount of CT rRNA detected between 2 and 25 hours p.i. when binary fission of RBs results in rapid expansion of the rRNA pool (Figure 16B). It has also been reported that RBs have up to three times more rRNA than EBs (Newhall

1988). The rapid expansion of the CT rRNA pool was followed by a three log increase in the amount of infectious CT which demonstrated that complete replication was occurring in untreated CT cultures. In penicillin-treated CT cultures, a similar trend was observed when cell-associated CT rRNA and CT recovery were compared. There was a marked decrease in the number of infectious CT early in the infection as EBs matured into RBs, while the concentration of CT rRNA increased dramatically until it peaked at 25 hours p.i. (Figure 17B). This dramatic increase in the levels of CT rRNA probably reflects binary fission of RBs as well as a greater concentration of rRNA per RB (Newhall 1988). The small peak of CT recovery at 32 hours p.i. temporally followed the peak of CT rRNA, as was observed in control cultures that suggests that this small peak of production was an abortive attempt at replication. Unlike control cultures, the peak of CT rRNA rapidly decreased over time although the levels remained well above all background controls (Figure 17B). This rapid decrease in the CT rRNA pool could reflect either downregulation of RB fission, downregulation of CT metabolism or destruction of CT. As the levels of CT rRNA remained well above background, it suggests that CT is harbored in these cells in some form, although RB fission may be slowed. It was not possible with this experimental design to determine if the levels of CT rRNA would continue to decrease with time, or would remain at a plateau, indicative of a persistent infection.

The kinetics of CT rRNA levels suggest that at approximately 25 hours p.i. some signal downregulated CT replication in penicillin-treated cultures. At the same time p.i. (25 hours p.i.) a three-log increase in cell-associated infectious CT was

observed in untreated cultures suggesting that EB maturation was occurring (Figure 17A). It has been reported that penicillin inhibits the synthesis of the 60 KDa cysteine-rich outer membrane protein which is an important wall component in CT EBs (Moulder 1991). This protein is extensively cross-linked and added to the EB cell wall with two other cysteine-rich proteins (12 and 59 KDa) during EB maturation (Moulder 1991). Penicillin may have multiple effects upon CT replication: a.) inhibition of the synthesis of a protein necessary for EB maturation, b.) inhibition of the extensive protein-to-protein cross-linking found in infectious EB cell walls and c.) inhibition of EB maturation resulting in feedback inhibition or downregulation of RB binary fission. Since CT contains three penicillin binding proteins found in CT, it would not be too improbable to postulate that more than one enzymatic reaction could be affected (Barbour et al. 1982). Spheroplast formation has not been discounted by these results nor has the definitive mechanism(s) of penicillin action upon CT replication been elucidated. However, if the hypothesis is correct, then penicillin treatment of CT-infected cells in vitro should result in a type of persistent infection.

Since the use of CH in this first set of experiments precluded examination of long-term persistence, another approach was employed. CT persistently-infected McCoy cells were cultivated either in the presence or absence of penicillin (100 U/ml). Unlike previous work (Lee and Moulder 1981, Benes 1990), no CH was added to the medium of the persistently-infected cell lines. Penicillin treatment of the initial passage resulted in abnormal inclusion formation consisting of vacuoles and incomplete inclusions which contained CT MOMP. With the exception of an occasional very small iodine-staining

inclusion, intracellular inclusion formation was not observed in the CT-persistently-infected McCoy cells while maintained in penicillin medium. As was expected, recovery of infectious CT from the PEN cell line rapidly decreased and was undetectable after passage 2. CT rRNA was detectable in passages 0-2 and at very low levels in passages 20-22 but was undetectable throughout all other passages (Figure 20). This does not imply that CT rRNA was not present. Rather this result may reflect that the representative sample size was too small (only 3.33% of the total lysate was assayed). Further work needs to be done to determine if CT rRNA and/or mRNA are present in the PEN cell line during passages 3-19. Although the PEN cell line was negative by the other parameters examined, passages 0-15 consistently harbored the CT genome, or at least a portion thereof, as the diagnostic 144 bp band was detected in amplification reactions using DNA extracted from the PEN cell line (Figures 26, 27, and 28). As had been hypothesized, penicillin treatment of CT-infected cells appeared to result in development of persistent infection, rather than elimination of CT.

Since false positive results are a chronic problem with PCR (Kwok 1990), more detailed examination of the CT-persistently-infected cell lines was required. Removal of penicillin immediately after the initial passage resulted in a productive CT infection with resumption of normal intracellular inclusion formation and recovery of infectious CT (Figure 21). As CT replication was never completely inhibited, it is not surprising that this cell line rapidly recovered with only one passage (P1) in which intracellular inclusion formation was not noted. This was in contrast to CT-McCoy cells cultivated in penicillin for 3, 6, 10 or 18 passages prior to cultivation in penicillin-free medium.

PEN_3 and PEN_6 became negative by the parameters examined yet "recovered" to establish a productive CT infection that had normal intracellular inclusion formation, high levels of CT rRNA detected and production of infectious CT (Figures 22 and 23). PEN_3 did not exhibit inclusion formation for 3 passages in control medium yet after subsequent cultivation reestablished a productive CT infection. The "recovery" of PEN_3 and PEN_6 substantiates the PCR results which demonstrated that the CT genome was harbored in the PEN cell line during these passages.

CT-McCoy cells grown in penicillin for 10 or 18 passages prior to cultivation in control medium reacted quite differently than PEN_1, PEN_3, or PEN_6. PEN_{10} developed low levels of very small iodine-staining inclusions from passages 12-20 (Figure 24). These inclusions did contain MOMP which indicates expression of the latter. These inclusions were small and pleomorphic. Ultrastructural analysis would be required to determine differences between the inclusions found in the PEN_{10} cell line and normal inclusions in untreated cells. Very low levels of CT rRNA were detectable in PEN_{10} cells, however these levels are far lower than those observed in active CT infection. No infectious CT was recovered from PEN_{10} from passages 10-20. PEN_{10} appeared to be a true persistent infection with low level antigen expression and low level replication of noninfectious CT, without production of infectious progeny. PEN_{18} exhibited a similar pattern as PEN_{10}, yet no iodine-staining inclusions were observed even though an occasional small inclusion was observed when the monolayers were stained with anti-MOMP (Figure 25). It is unknown if further cultivation of PEN_{10} and PEN_{18} in penicillin-free medium would have resulted in spontaneous reactivation to a productive

infection. Although these results support the PCR findings that the CT genome is harbored in the PEN line, it is unknown if the CT DNA harbored in these cells was damaged so that reactivation was impossible or if some required signal for reactivation was absent from the in vitro environment. In this regard, it has been reported that high levels of endogenous cyclic AMP can reactivate CT-persistently-infected BHK cells (MacDonald et al. 1986). Further studies of CT-McCoy cells are needed to determine if the CT DNA harbored in the cells treated long-term with penicillin becomes irreparably damaged, and to dissect the various signals necessary for reactivation to productive infection.

It is also unknown in what form CT is harbored in persistently-infected cells. The form appears to be noninfectious as it can not be detected by culture. It would seem reasonable that RBs are harbored, yet spheroplast formation has not yet been ruled out. Since infectious CT is not produced in penicillin-treated CT cultures, then some other mechanism of cell to cell transfer must be used. Transfer of chlamydial inclusions to daughter cells at cell division has been reported (Ward 1988). Transfer of the harbored CT to daughter cell(s) appears to have occurred since CT DNA was detected by PCR in CT-McCoy cells that had been cultivated in the presence of penicillin for 19 passages, even though no infectious CT had been detected for months in this cell line. Thus the CT genome appears eventually to be diluted out. This suggests that the level of replication in the PEN line was very low and that progeny may not be passed to both daughter cells upon division. However, it has not yet been determined if the harbored form of CT was undergoing low level replication in the presence of penicillin or if it was

in some dormant state, i.e. a true latent infection. Molecular techniques employing antisense probes and in situ hybridization would be required to answer this question.

Although considered a rare epidemiologic event, there have been reports of CT isolates resistant to multiple antibiotics including doxycycline, erythromycin, sulfamethazole, clindamycin and tetracycline (Jones et al. 1990, Mourad et al. 1980). Examination of the PEN_I cell line and its sensitivity to penicillin was necessary to determine if "recovery" to productive infection was due to selection of penicillin-resistant strains of CT. As PEN_I appeared to be fully susceptible to penicillin, reactivation of the persistent infections did not appear to be reliant upon selection of penicillin-resistant strains of CT. However, since no cloning studies (including susceptibility assays of clones derived) were performed, it is not possible to state that no penicillin-resistant strains ever developed during this study.

Several other lineages of CT-McCoy cells were developed without the use of penicillin. McCoy cells inoculated with a high MOI of CT underwent the "wipeout" described by Lee and Moulder (1981), with cycles of cell lysis followed by regeneration of the cell monolayer. There were several basic differences between the current study and earlier work reported. In earlier work, after the initial "wipeout", 100-150 days elapsed before productive infection was noted (Lee and Moulder 1981). The CT-McCoy cells generated without penicillin in our study remained overtly infected but did undergo episodes of cellular destruction and regeneration. In earlier work, addition of penicillin (16 U/ml) to CT-persistently-infected McCoy cells for 5 days--with subsequent cultivation in penicillin-free medium--did not inhibit inclusion formation although it did

delay the destructive process (Lee and Moulder 1981). In the current study, addition of penicillin (100 U/ml) to CT-McCoy cells developed in penicillin-free medium resulted in total abrogation of inclusion formation with only a low level of infection noted in the treated cells when they were subsequently cultivated in control medium. Although both studies used "McCoy" cells, there are many differences between McCoy cells from different sources. The discrepancies observed between the current study and earlier work could reflect differences between the cell lines employed, the use of CH, or differences between the strains of CT employed. In fact, by using a very low MOI, our McCoy cells could be persistently infected with CT without the cycles of destruction reported elsewhere (Lee and Moulder 1981, Benes 1990).

In another study, using CT-inoculated BGM cells, it was shown that addition of antibiotics 2 days p.i. resulted in neither abrogation of inclusion formation nor inhibition of infectious CT production, although both were decreased (Clad et al. 1990). The antibiotics tested were doxycycline, erythromycin, ciprofloxacin, and ampicillin (Clad et al. 1990). Once again this was in contrast to the current study in which addition of penicillin to persistently-infected McCoy cells completely halted inclusion formation and production of infectious CT for several passages, with productive infection developing after subsequent cultivation in control medium. Further studies are needed to determine why these discrepancies have occurred, and what the actual mechanism(s) are that control CT persistence and antibiotic susceptibility.

As has been noted previously, reported MIC values for CT vary widely depending upon the parameter assayed (Blackman et al. 1977, Lee et al. 1978, Hobson et al. 1982,

How et al. 1985). In vitro antimicrobial susceptibility testing is considered an acceptable method for determination of a microorganism's sensitivity to different antibiotics. In the case of CT, however, this method may not necessarily be valid. Although penicillin is not the treatment of choice for CT infections in vivo, other β-lactams are considered efficacious due to in vitro susceptibility testing and some human drug studies (Martin et al. 1986). Although the cases may not be exactly analogous, the finding that several of the CT-McCoy cells of the penicillin lineage remained negative by all parameters tested for several passages prior to becoming overtly infected suggests that in vitro susceptibility testing of CT may need to be examined more thoroughly. Examination of several parameters may be necessary to determine the true efficacy of different antibiotics in vitro, including more than just a single passage in tissue culture. It may also be necessary to utilize molecular techniques to differentiate between development of persistent infection and true eradication of CT.

The effect of penicillin upon CT replication has been used as a tool to examine one type of persistent infection at the molecular and cellular level. Penicillin may not induce persistent infections in vivo or the immune response may remove persistently-infected cells. However, the type of abnormal inclusion observed with penicillin treatment has also been observed when CT-infected cells are exposed to interferon-γ (Beatty et al. 1991). It may be that a number of cellular or molecular different signals can result in development of persistent infection, possibly by downregulation of EB production and RB binary fission until a reactivation signal is provided. The fact that nutrient deprivation can also result in "delayed" CT infections may help to support this

speculation, but additional work is needed to resolve this issue.

The significance of persistent in vitro CT infections has been debated. Although valuable model systems for controlled study of cellular and molecular mechanisms, in vitro model systems can not encompass the complex immune response that occurs in vivo. For persistent infections to occur in vivo, a successful host cell-parasite relationship must be established and the infected cells must evade destruction by the immune responses as well. To examine persistent CT infections in vivo, a murine model of upper tract CT genital infection was used. Earlier work reported that progesterone pretreatment of female C3H mice enhanced subsequent CT infection (Tuffrey et al. 1986a). The murine model of CT salpingitis has become widely used to study adverse sequelae of severe upper tract CT infection (Tuffrey et al. 1990, Swenson et al. 1986, Patton 1990, Zana et al. 1991). In experiment 1, mice which had not been pretreated with progesterone transiently shed CT vaginally, although 50% of the mice harbored CT in the upper reproductive tract tissues (Table 2). All of the nontreated mice were sacrificed relatively early p.i. (15 and 43 days). It was notable that one mouse had not shed CT vaginally for over a month yet still harbored CT in the upper tract. Thus in nontreated CT-inoculated mice, lack of vaginal CT shedding did not reflect true eradication of the infection from the upper tract (Table 2).

DP-pretreatment enhanced vaginal CT colonization as CT was recovered with greater frequency from successive vaginal swabs (Table 4). This result agrees with our earlier work in which DP-pretreatment enhanced vaginal shedding of CT and dissemination of CT throughout the upper reproductive tract (Tau-Cody et al. 1989). An

unexpected result was that a number of the DP-pretreated CT-inoculated mice underwent recurrent vaginal shedding of CT, which indicated that even successive negative vaginal cultures did not correlate with clearance of CT from the upper tract (Table 4). It has been accepted by some that successive negative vaginal CT cultures correlates with resolution of the infection (Igietsume et al. 1991). This result brings that assumption into question. Early in the infection (15 days p.i.), 83% of the DP-pretreated mice were not shedding CT vaginally, yet all CT-inoculated mice sacrificed at that timepoint harbored CT in multiple tissues of the upper tract. This further supports the finding that CT can be sequestered in the upper tract, undetectable by standard assay techniques which rely upon cervical or vaginal specimens. In our study, CT was not isolated from the vaginal secretions or from upper tract tissues of any of the mice sacrificed late in the infection (84 days p.i.). Several other studies have indicated that mice sacrificed late in the infection (19 to 21 weeks p.i.) appear to have effected a bacteriologic cure since CT was not isolated from the upper tract tissues (Zana et al. 1990, Zana et al. 1991). It has not yet been determined if these mice had indeed eradicated the infection or if these mice harbored CT in a persistent infection undetectable by culture. Investigations employing molecular techniques are required to answer this question.

In the current study, gross pathologic evidence of infection was observed in untreated and DP-pretreated mice. Post-surgical adhesions were seen frequently in mock-inoculated and CT-inoculated mice, underlining a drawback of this inoculation technique. This murine model employs a surgical inoculation into the tip of the right uterine horn which is not the natural mode of infection, generating a descending infection rather than

the natural ascending infection. This liability must be considered when scoring gross pathology, as some damage may be due to the trauma of surgery, not to CT infection. Hydrosalpinx formation, erythema and enlarged fallopian tubes were observed only in CT-inoculated mice and were scored as CT-specific gross pathology. In mice that were not pretreated with DP, isolation of CT from the upper tract tissues did not correlate with detectable presence or absence of gross pathologic damage (Table 2). Some mice which had evidence of gross pathologic damage did not appear to harbor infectious CT (Tables 2 and 4). The mechanisms by which CT pathogenesis is produced are poorly understood at this time. Several mechanisms have been suggested and the damage caused by CT infections may be produced by more than one mechanism. As some untreated mice exhibited evidence of gross pathologic damage without apparent infectious CT present, more than one mechanism may be involved . One possibility is that the damage may have occurred prior to elimination of the infection. This hypothesis can neither be proved nor disproved by the results of this experiment as: a.) it has not been definitively determined whether or not CT has been eliminated from these mice and b.) the specific time at which the damage occurred has not been determined. In general, gross pathologic damage was observed in nontreated CT-inoculated mice by 6 weeks p.i., but was observed in DP-pretreated CT-inoculated mice as early as 1 week p.i. (Tables 2 and 4). "Irreversible" damage may be caused by or associated with CT replication. When CT is eliminated, the damage would persist. Several other mechanisms of pathogenesis have been suggested. Hypersensitivity has been implicated as one mechanism of CT pathogenesis. The most direct evidence has been the purification of a 57 KDa CT

protein which elicits a delayed hypersensitivity reaction in the ocular model (Morrison et al. 1989). The role of immunopathogenesis is further implicated by studies using the MoPn strain of CT in a model of reactive arthritis. It has been demonstrated that reactive arthritis can be induced in C57Bl/6 mice provided that the mice were sensitized by immunization or genital inoculation of MoPn prior to homologous articular challenge. The pathogenesis apparently occurred without active CT replication (Hough and Rank 1988, Hough and Rank 1989). Another model examining salpingitis in CBA or C3H mice showed that repeated inoculations of CT resulted in greater severity of salpingitis, even though vaginal CT shedding was less prolonged or did not occur in the challenged mice (Tuffrey et al. 1990). Evidence from earlier reports and from the current work strongly suggest that tissue damage can occur in the absence of apparent CT replication and that the immune response to CT infection may be both protective and destructive. It has also been reported that tetracycline treatment during the first week of infection could prevent subsequent development of infertility in CT-inoculated mice but treatment given after 1 week p.i. had little protective effect (Swenson et al. 1986). This suggests that even successful antibiotic treatment that halts CT replication may not prevent subsequent sequelae from developing. A variation on the theme of CT immunopathogenesis is that the mice become CT-persistently-infected in vivo. The chronic inflammatory reaction thus induced eventually leads to tissue damage, either through the presence of inflammatory cytokines (TNF-α), through scarring or deciliation of the tubal mucosa or through other immunopathogenetic mechanisms, such as autoimmunity. Histopathologic examination of the tissues from the nontreated CT-

inoculated mice in our study may help explain the results observed by detailing the presence and type of microscopic damage.

Similar results were obtained when the gross pathology of DP-pretreated CT-inoculated mice was examined. At the time of sacrifice 73.3% of the mice exhibited gross pathologic evidence of CT-specific damage (Table 4). As DP-pretreatment seemed to enhance dissemination and replication of CT in mice, many mice sacrificed during the timecourse of the experiment were still CT positive at the time of sacrifice. Of the 3 mice sacrificed at day 84 p.i. one had a hydrosalpinx in the absence of either a positive vaginal or upper tract CT culture. An analogous argument can be made for the immunopathogenetic response to CT infection in DP-pretreated mice, however the effect of DP upon this response has not yet been determined. Histologic examination of the tissues from the DP-pretreated CT-inoculated mice must be performed to determine the presence and type of damage. These results must then be compared to the type of damage that had occurred in nontreated CT-inoculated mice to determine if DP had altered this parameter of infection.

Further analysis of the results from experiment 1 revealed that a valuable model to examine CT persistent infection in vivo would be inoculation of C3H mice without DP-pretreatment followed by observation and sampling at 2-6 weeks p.i. Although these mice only transiently shed CT vaginally, CT was harbored in the upper tract tissues of 50% of the mice and gross pathologic evidence of infection was observed (Table 6). This model may prove to be the most productive for investigation of the effect of various antibiotics upon "persistent" CT infection in vivo, i.e. whether or not the antibiotic under

investigation truly eradicates CT infection or results in the establishment of a persistent infection.

Since penicillin treatment of CT-infected McCoy cells resulted in establishment of persistent CT infection in vitro, the effect of penicillin upon CT infection in vivo was examined in experiment 2. The penicillin regimen employed was i.m. injection of 2000 U of penicillin G at days 9 and 16 p.i., a dosage that was equivalent to 2,000,000 U in an average size human. Unexpectedly, penicillin treatment of DP-pretreated CT-inoculated mice did not halt vaginal shedding of CT (Table 7). This unexpected result may be due to several factors. It has been reported that the serum half life of amoxycillin and clavulanic acid (both β-lactams) in mice was 1/2 to 1/10 as long as the serum half lives of the same two antibiotics in humans (Beale et al. 1991). It is possible that the treatment regimen employed did not allow sufficiently high concentrations of penicillin to be achieved for a duration long enough to affect CT replication. Another factor may be the genital bioavailability of the penicillin G solution injected. The latter was not examined. Injection of penicillin did alter the pattern of vaginal CT shedding as only 64.7% of the penicillin-treated mice exhibited recurrent vaginal shedding compared to 90.3% of the unmedicated CT-inoculated mice (Table 7). More detailed analysis of the results revealed that at 2 weeks p.i., only 16.7% (experiment 1) and 19.4% (experiment 2) of the unmedicated CT-inoculated mice were vaginally shedding CT as compared to 62.5% of the penicillin-treated CT-inoculated mice at the same timepoint (Table 8). None of the nontreated, unmedicated, CT-inoculated mice were shedding CT vaginally at 2 weeks p.i. At 2 weeks p.i. some critical event appears to

have occurred which resulted in the apparent "elimination" of CT from the vaginal vault of unmedicated CT-inoculated mice. At this time it is unknown what caused this "elimination" and why it would be modified by the penicillin treatment employed. It is possible that penicillin in vivo induces a persistent or sequestered CT infection in some form which escapes the immune response, but this is speculation.

At 10 weeks p.i., mice which had been CT-negative for $\geq$2 successive cultures were induced with CA or DP in an attempt to reactivate vaginal CT shedding. Mice from the same subpopulation were injected with saline to serve as controls. CA had been demonstrated to reactivate CT lung infections in mice (Yang et al. 1983). As DP-pretreatment has been demonstrated to enhance the susceptibility of mice to CT genital infection, it was tested to ascertain if it also could reactivate vaginal CT shedding. Penicillin treatment did reduce the number of mice that underwent reactivation of vaginal CT shedding as only 1/7 mice injected with CA had a transient reactivation of vaginal CT shedding, while none of the mice injected with either DP or saline became CT-positive (Table 9). Of the unmedicated CT-inoculated mice that underwent induction, 28% injected with CA and 33% injected with DP had a transient reactivation of vaginal CT shedding, while none of the mice injected with saline became positive for CT (Table 9). These reactivation events were transient and only low numbers of inclusions were observed. These results suggest that penicillin treatment reduced the number of mice in which a persistent CT infection was established which could be reactivated by CA or DP, but further work is needed to determine this. In the model of CT lung infection reported, only 33% of the mice that underwent steroid induction experienced reactivation

of the CT infection (Yang et al. 1983). It is possible that only a portion of the mice inoculated with CT actually develop a chronic persistent CT infection or it may be that these induction protocols did not provide the most efficient reactivation signals. It could be important to determine what events caused the persistent infection only in some mice, since the inbred mice employed in this study are 99.99% genetically identical. Understanding differential response to CT infection among individuals with the same genetic background may allow better comprehension of CT pathogenesis overall. No infectious CT was recovered from the upper tract tissues of any animal in the first reactivation group at the time of sacrifice (16 weeks p.i.). As noted before, it is unknown at this time if CT had been truly eliminated from these mice or was simply in a form of persistent infection which was undetectable by culture. A second subpopulation of mice from this experiment underwent the same induction regimen as described except induction began at 17 weeks p.i. None of the mice from any group became CT-positive and infectious CT was not recovered from any animal at the time of sacrifice (22 weeks p.i.). Further analysis of the tissues from these mice using molecular methods such as in situ hybridization or PCR must be performed to determine if CT had been eradicated from these animals or remained sequestered in a persistent or latent form.

Gross pathologic evidence of tissue damage was observed at the time of sacrifice in 70.6% of the penicillin-treated mice and 71.0% of the unmedicated mice, even though infectious CT was not recovered from any of these animals (Table 7). This supports earlier findings that active CT replication may not be necessary for pathogenesis.

Penicillin treatment did not protect the CT-inoculated mice from this damage. This lack of protection by penicillin may be the result of an inefficacious therapeutic regimen, or it may be due to the late start of antibiotic therapy (9 days p.i.), as was seen in a mouse model mentioned earlier, in which tetracycline treatment started after 1 week p.i. did not protect against subsequent infertility (Swenson et al. 1986). Histologic examination of the tissues from these mice must be performed to answer some the unresolved questions from this experiment.

As with in vitro testing of CT susceptibility to various antimicrobial agents, the ability of CT to establish undetectably persistent infections in vivo may require a closer examination of results obtained from animal models and human studies of CT antibiotic susceptibility. Traditionally these studies rely upon cervical or vaginal elimination of CT as test of cure. Antibiotic susceptibility assays in animal models allow for examination of upper tract tissues by molecular methods to determine if CT had been cleared by an antibiotic or simply forced into a persistent infection undetectable by commonly used assays. With human drug studies, however, the question can not be addressed as directly. All such studies use elimination of CT from cervical specimens as test of cure. This is necessary as laparoscopy, which would allow tubal biopsies to be obtained for further analysis, is an invasive procedure that should not be performed unless warranted. At this time, laparoscopic examination of all women undergoing treatment for CT to determine efficacy of cure is unreasonable. However, the problem does exist. In one study, infertile women underwent laparoscopy and tubal biopsies from some of these women were CT positive, even though the women had negative endocervical CT cultures

(Henry-Suchet 1988). Serology is not diagnostic of persistent CT infection as CT-specific antibodies can persist for years (Mardh et al. 1989). Understanding of the mechanism(s) of CT persistence may allow for better assessment of what factors may put a person at risk of establishing a persistent infection, which if not treated correctly, may be reactivated to cause eventual damage.

A better understanding of the protective and pathogenic aspects of the immune response to CT, the nature of persistent or latent CT infections in vivo and the response of CT to various antibiotics could be vital. With in vitro and in vivo models now available to examine these questions at the molecular level, it could become possible to better assess which women may be at risk for the more serious sequelae of CT infection and permanent tissue damage. Among the unresolved questions concerning CT pathogenesis, the role of CT persistence and/or latency may prove pivotal, as so many critical decisions are based upon the assumption that a negative cervicovaginal specimen correlates to clearance of the infection. Persistent CT infections could force a reevaluation of many assumptions and have a significant impact upon the clinical management of this infection.

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